Molecular analysis of halorespiration in *Desulfitobacterium* spp.

– CATALYSIS AND TRANSCRIPTIONAL REGULATION

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Molecular analysis of halorespiration in *Desulfitobacterium* spp.

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ABSTRACT

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Soil and ground water contamination by halogenated organic compounds mainly used as biocides in agriculture or solvents and cleaning agents in industry has been a long-standing problem. The main barrier in the chemical degradation of organohalides is the presence of the halogen group in the molecule, which also contributes to the toxic nature of these compounds. However, a remarkable group of microorganisms, the halorespiring bacteria, are able to reductively dehalogenate organohalides under anaerobic conditions and use the energy generated via a proton gradient pump for bacterial growth (halorespiration). Due to their versatile dehalogenating capacity, halorespiring bacteria have a large potential in the clean-up of contaminated sites (bioremediation). The work described in this thesis aimed to gain knowledge on two aspects of the molecular basis of halorespiration: (i) the reaction mechanism of dehalogenation, catalyzed by the B_{12}/iron-sulphur containing reductive dehalogenases, and (ii) the regulatory mechanism which enables transcriptional activation of genes involved in halorespiration. As model organisms, two *Desulfitobacterium* spp. were chosen, capable of *ortho* and/or *meta* dechlorination of chlorophenols and hydroxylated polychlorinated biphenyls.

Unravelling of the novel reaction mechanism of reductive dehalogenases was so far hampered by difficulties in functional overproduction of these enzymes. We developed a protocol which involves co-expression of molecular chaperons to aid functional synthesis of the metalloenzymes in *Escherichia coli*. Next, multiple potential transcriptional activators were characterised from *D. hafniense* by promoter fusions and *in vitro* DNA-binding assays. We found that CprK1 and CprK2 activated transcription in the presence of an *ortho*-chlorophenol (CHPA) but not with its dechlorinated derivative (HPA), while *meta*-chlorophenols proved to be effectors for CprK4. All CprK paralogues recognized a conserved motif (dehalobox) in halorespiration-inducible promoters. Site-directed mutagenesis of CprK1 provided further insight on the role of conserved residues in the DNA-recognition α-helix and on redox regulation of the protein.

Crystal structures of the CHPA-bound CprK1 and the effector-free form of the closely related CprK of *D. dehalogenans* revealed a possible mechanism by which the regulators distinguish between CHPA and the non-effector HPA ("pK_a interrogation" theory). Native mass spectrometry of protein-DNA complexes confirmed these results and – together with limited proteolysis experiments – also contributed to the fundamental knowledge of ligand-induced changes in the conformation and dynamics of CprK-related transcriptional regulations.

Keywords: iron-sulphur proteins, protein-DNA interaction, allosteric regulation, redox regulation, gene redundancy, chlorophenols, bioremediation

CONTENTS OF THE THESIS

PREFACE

Dioxins, DDT, and polychlorinated biphenyls (PCBs) are compounds with a negative connotation that belong to the group of persistent organic pollutants. They are suspected to be responsible for birth defects, immune system dysfunction, and reproductive problems in wildlife, deserving the name "dirty dozen" together with nine other organic compounds. They are not only toxic, but also very persistent against chemical degradation, due to the high degree of halogen substitution within the molecules. Therefore the discovery that anaerobic bacteria can degrade halogenated organic compounds in a respiratory-type mechanism was of utmost significance. These bacteria can replace the halogen atom(s) in the molecule with hydrogen, which often renders a less toxic or more bioavailable end-product, and conserve energy from the reaction via electron transport-coupled phosphorylation in a process termed halorespiration. The genus *Desulfitobacterium* constitutes a prominent group of halorespiring bacteria, including several isolates that can couple dehalogenation of *ortho*- and/or *meta*-chlorophenols and hydroxylated PCBs to energy generation and growth. The dechlorination step is catalyzed by cobalamin/iron-sulphur containing enzymes, the reductive dehalogenases, which are almost exclusively produced when their halogenated substrates are present in the environment.

The aim of the research described in this thesis was to gain knowledge on the molecular basis of halorespiration. By recognizing the most important factors involved in catalysis and activation of reductive dehalogenase gene expression, it is anticipated that the biodegradation capacity of halorespiring bacteria can be efficiently enhanced. We focused on two main topics: (i) to unravel the reaction mechanism that underlies the carbon-halide bond cleavage by CprA, an *ortho*-chlorophenol reductive dehalogenase from *Desulfitobacterium dehalogenans*; and (ii) on the characterisation and structure-function analysis of CprK1, a putative transcriptional regulator of halorespiration in *Desulfitobacterium hafniense*. Outline of the content of the thesis:

- ***Chapter 1*** gives a general overview on halorespiration and related fields, including the production of halogenated organic compounds, the diversity of halorespiring bacteria, the isolation and characterisation of reductive dehalogenases and the CRP-FNR superfamily of transcriptional regulators, with special attention on structural features that influence the biological function of these regulators.
- ***Chapter 2*** describes the characterisation of CprK1, a new member of the CRP-FNR family. Promoter fusion and *in vitro* DNA-binding experiments were used to find the effector molecules that activate CprK1 and enable it to bind to specific DNA sequences in halorespiration inducible promoters. Site-directed mutagenesis of CprK1 revealed details on the specific recognition of its DNA target and on putative redox regulation of the protein.
- ***Chapter 3*** provides further details on the allosteric effects of chlorophenol binding to CprK1 by describing the crystal structure of the effector-bound CprK1 and that of the closely related CprK from *D. dehalogenans* in its effector-free form.

- ***Chapter 4*** reports on the structural dynamics and ligand-induced conformational changes of CprK1 using macromolecular mass spectrometry, limited proteolysis coupled to mass spectrometry and *in vitro* DNA-binding assays in the presence of several potential effector molecules.
- In ***Chapter 5***, four *cprK* gene homologues are described that are present in the almost complete genome sequence of *D. hafniense*, additionally to the CprK1-encoding gene. To determine whether their redundancy causes merely a dosage effect or their function is diverged and specialized, we overproduced three of the CprK paralogues in *E. coli* and studied transcriptional regulation mediated by the heterologously-produced proteins *in vivo* and their DNA-binding properties *in vitro*.
- ***Chapter 6*** focuses on the catalytic basis of halorespiration, and aims to provide details of the yet-unknown reaction mechanism. The first step towards this aim is the overproduction of functional reductive dehalogenases in genetic model systems, for which we selected *E. coli*. Our attempts included the improvement of anaerobic culturing conditions and the co-expression of molecular chaperons and a trigger factor to aid correct protein folding.
- Finally, in ***Chapter 7*** (in English) and in ***Chapter 8*** (in Dutch) the main findings presented in this thesis are summarized and evaluated with respect to the implications of the work to the fundamental knowledge on halorespiration and to the practical aspects of bioremediation.

1

General introduction

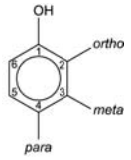
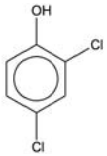
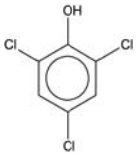
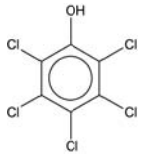
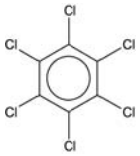
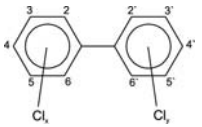
Halogenated hydrocarbons

Halogenated organic compounds are known to be present in the environment due to their natural production (Gribble, 1994). The number of known, mainly chlorinated and brominated hydrocarbons comprises currently over 3800 compounds, which can be produced either abiotically (due to volcanic activities, forest fires) or biosynthesized by marine algae, bacteria, terrestrial plants, fungi and insects (Table 1.1). Since the last century, the extensive anthropogenic production of mainly chlorinated aromatic and aliphatic compounds has been substantially interfering with the existing natural chlorine cycle (Oberg, 2002). The initial enthusiasm for using haloorganic compounds as biocides, solvents, flame retardants and in various other applications (Table 1.1) resulted in the production of large amounts of hazardous halogenated organic waste, which was often not properly disposed, causing severe soil and ground water contaminations. Because of their physicochemical properties (Table 1.2), exposition to halogenated organic compounds has potential or proved negative health effects (Table 1.1). Therefore, attempts were made to provide "green" alternatives for these compounds (e.g. replacing chlorofluorocarbons in refrigerators with liquefied propane) or to regulate their selective collection and proper waste deposition. These efforts are now aided by the “Montreal Protocol” – an international treaty signed in 1987 –, and by supra-governmental regulations such as the EU Council Directive 91/689/EEC on hazardous waste. In the Netherlands, the importance of soil remediation has been recognized not only in the Soil Protection Act (1994) but also by investing 1.4 billion euros to solve this serious problem by 2022 (VROM, 2006).

Remediation strategies

Soil remediation processes may involve physicochemical treatments such as thermal cleaning or the extraction of the contaminants using evaporation followed by adsorption on activated carbon (De Best, 1999; Van Eekert *et al.*, 2001). However, these technologies are usually inefficient, costly and result in the destruction of the soil structure (Paul *et al.*, 2005). A viable alternative for soil clean-up is bioremediation, a process where contaminants are degraded by microorganisms or higher organisms like trees and plants (phytoremediation). Biodegradation of halogenated compounds by microorganisms can occur in four different ways: i) the halogenated compound can serve as a carbon source and oxidisable electron donor with either oxygen or other alternative compounds (nitrate, Fe^{III}, sulphate or humic acids) as an electron acceptor (Dijk *et al.*, 2003); ii) fermentative metabolism; iii) halorespiration, where the halogenated compound serves as terminal electron acceptor coupled to the oxidation of an electron-rich compound; and iv) co-metabolic dehalogenation due to non-specific activity of usually transition metal-containing enzymes that are involved in other physiological processes (Janssen *et al.*, 2001). Although many halogenated pollutants can be degraded under aerobic conditions, often by members of the versatile *Pseudomonas* genus (Lovley, 2003), the more halogen substitutions these compounds contain, the more recalcitrant they become to oxygen-aided degradation (Janssen *et al.*, 2005).

Table 1.1 Ecological and biological impact of organohalides

	ortho-, meta-, para-CP [a,b]	2,4-DCP [b,c]	2,4,6-TCP [b,c]	PCP [a,b]	PCE [d]	HCB [a]	PCBs [a]
Structure							
Occurrence	Mostly bound to soil particles, adsorption is strong in acidic, organic soils, but low in basic, mineral soils.				Mostly in vapour phase	Mostly in vapour phase	Lipophilic, semi-volatile, mostly adsorbed to particles in air, soil.
Abiotic degradation	Photodegradable ($t_{1/2}$ varies from hours to days)	Degradation in minutes by UV irradiation in aqueous solution.	Degradation by UV irradiation on silica gel ($t_{1/2}$ 17 h).	Photodegradable ($t_{1/2}$ 14 days in soil).	Persistent (no photodegradation in water).	Persistent ($t_{1/2}$ is 1.5-2 years in the atmosphere).	Persistency increases with halogen substitution ($t_{1/2}$ between 10 days to 1.5 year).
Natural production [e]	Metabolites of microbes, fungi (2,4-DCP is produced by *Penicillium* sp.), ticks (2,6-DCP secreted as sex pheromone), and grasshoppers (2,5-DCP as repellent); can occur in proteins (chlorinated tyrosine in proteins from marine sponges).				Volcanic activity, barley	Halogen containing minerals	Indirectly by volcanic activity
Anthropogenic production	Wood preservation, inhibitors of microbial growth in oils, textiles, and pharmaceutical products.	Intermediate in 2,4-D synthesis, used as repellent in mothballs.	Germicide, preservation of wood, leather, glue, and textiles.	Wood preservation, by-product of water treatment.	Solvent in metal and dry cleaning, used in grain fumigation.	By-product in pesticide synthesis, intermediate in dye production.	Dielectric and heat-exchange fluids, also used in microscope oil.
Toxicokinetics	Readily absorbed across the skin and the gastrointestinal tract, accumulation in liver and kidney. Detoxification by conjugation to glucuronates or sulphates in the liver.				Rapid absorption by skin or lung, accumulates in fatty tissues. Catabolized via the cytochrome P450 oxidase system.	High absorption by the digestive tract, low dermal intake. Mainly catabolized in the liver.	Rapidly absorbed in fatty tissues.
Systemic effect	Generally, toxic effect of chlorophenols involves the uncoupling of mitochondrial oxidative phosphorylation.	Increased spleen and liver weights and haematological or immunological effects, one death incidence after dermal exposure.	Leukaemia and liver cancer in rats.	Alterations in hepatic enzyme activities, increased liver weight, histopathological and carcinogenic effects.	Dermal irritation, liver and kidney damage, alterations in metabolism, long term neurotoxic effect, risk of cancer.	Effect on immune function, liver activity, cancer, death (Turkey 1955-1959).	Induction of cytochrome P450 (ligand for AhR) [f], endocrine disturbances (ligand for TR) [g], respiratory tract toxicity, cancer.

[a] (WHO, 2003); [b] (WHO, 1989); [c] (ATSDR, 1999); [d] (WHO, 1984); [e] (Gribble, 1994); [f] reported for dioxins; [g] (Schriks *et al*., 2006). Abbreviations: CP, chlorophenol; DCP, dichlorophenol; TCP, trichlorophenol; PCP, pentachlorophenol; PCE, tetrachloroethene; HCB, hexachlorobenzene; PCBs, polychlorinated biphenyls; 2,4-D, 2,4-dichlorophenoxyacetic acid; AhR, aryl hydrocarbon receptor; TR, thyroid hormon receptor.

This is caused by the electronegative nature of the halogen atom, which hampers the oxygen attack on the carbon backbone of the haloorganic compound (Wohlfarth *et al.*, 1997). Conversely, halogenated hydrocarbons with a high degree of substitution are generally more readily degraded under anoxic conditions. This and the fact that most of the contaminated sites such as aquatic sediments, submerged soils and ground water are oxygen depleted, makes anaerobic bacteria that are capable of halorespiration, good candidates for bioremediation (Van Eekert *et al.*, 2001).

Halorespiration

Halorespiration is a type of anaerobic respiration in which a chlorinated compound is used as a terminal electron acceptor, while energy is conserved via electron transport-coupled phosphorylation (Holliger *et al.*, 1998; Smidt & de Vos, 2004). During the reductive dehalogenation reaction, one or more chlorine atoms are removed from the molecule and replaced by hydrogen (hydrogenolysis) or in the case of saturated aliphatic organohalides, two neighboring halogen substituents can also be simultaneously removed, resulting in the formation of a double bond (dichloroelimination) (Chen, 2004; Van Pee *et al.*, 2003). Organohalides are excellent electron acceptors, as the standard redox potential for most R-Cl/R-H couplets lies between approximately +250 and +600 mV, in the range of the redox potential of the NO_3^- / NO_2^- couplet (E_0' = +433 mV) (El Fantroussi *et al.*, 1998). This ensures that reductive dehalogenation is a thermodynamically favourable, exergonic reaction, yielding between -130 and -180 kJ/mol of Gibbs free energy per chlorine removed by hydrogenolytic reductive dehalogenation, enabling bacteria to couple reductive dehalogenation to their growth (Smidt & de Vos, 2004).

Table 1.2 Physicochemical properties of selected organohalides

	2,3-DCP [h]	3,5-DCP [h]	2,4,5-TCP [h,i]	CHPA	2,4-DCP [h,i]	2,4,6-TCP [h,i]	PCP [h,j]	PCE [h,k]	HCB [h,j]	PCBs [j]
Molecular weight	163.00	163.00	197.45	186.6	163.00	197.45	266.35	165.82	284.79	various
Water solubility (mg/L)	905 [a]	39.9 [a]	380 [a]	freely soluble	1910 [a]	243 [a]	14 [b]	150 [b]	0.005 [c]	1.08×10^{-5} to 9.69×10^{-10} mol/L
Log K_{OW}	3.00	3.44	3.61	n.d.	2.96	3.61	3.32	2.89	5.61	4.46 to 8.18
Log K_{OC}	2.56	2.34	3.36	n.d.	2.54	3.47	4.5	2.86	5.5	5.6 to 8.0 [g]
E_{LUMO} (eV) [l]	n.d.	n.d.	n.d.	n.d.	-0.427	-0.821	-1.431	n.d.	n.d.	n.d.
Vapour pressure [c]	12.7 Pa	2.46 Pa	1 Pa	n.d.	4.09 Pa	0.983 Pa	0.00011 mmHg	1.9 kPa [b]	0.0023 Pa	5.6×10^{-12} to 4×10^{-9} mmHg [g]
Dissociation coeff. (pK_a)	7.66	8.17	7.07	8.4	7.81	6.22	4.85	-	-	-
Henry's law constant [d]	9.76×10^{-4} [e]	4.26×10^{-3} [e]	5.1×10^{-6}	n.d.	$4,3 \times 10^{-6}$	5.7×10^{-6}	3.4×10^{-6}	9.29×10^{-1} [e]	131 [f]	0.3×10^{-4} to 8.97×10^{-4}

[a] at 10°C; [b] at 20°C; [c] at 25°C; [d] Henry's law constant is given in atm m^3/mol at 25°C; [e] dimensionless Henry's law constant; [f] Henry's law constant is given in Pa × mol/ m^3; [g] values are for dioxins; [h] (Otte *et al.*, 2001); [i] (ATSDR, 1999); [j] (WHO, 2003); [k] (WHO, 1984); [l] (Lu *et al.*, 2001). Abbreviations: DCP, dichlorophenol; TCP, trichlorophenol; CHPA, 3-chloro-4-hyroxyphenylacetic acid; PCP, pentachlorophenol; PCE, tetrachloroethene; HCB, hexachlorobenzene; PCBs, polychlorinated biphenyls; K_{OW}, octanol/water partition coefficient; K_{OC}, organic carbon partition coefficient; E_{LUMO}, energy of the lowest unoccupied molecular orbital; n.d., not determined.

Halorespiring bacteria

The first anaerobic bacterium to be isolated that is able to couple reductive dehalogenation of a chlorinated compound, i.e. 3-chlorobenzoate to ATP synthesis was *Desulfomonile tiedjei* (DeWeerd *et al.*, 1991). Since then, more than 30 halorespiring bacteria have been isolated from very different pristine and polluted environments, such as activated and anaerobic granular sludge, freshwater- and estuarine sediments (Smidt *et al.*, 2000a). These isolates belong to different branches of the phylogenetic tree, based on their 16S rRNA sequence, namely to the gram-negative δ- and ε-proteobacteria or to the low G+C content gram-positive bacteria (Fig. 1.1). Exceptions are members of the *Dehalococcoides* genus, since these are most closely related to the green non-sulphur bacteria (Cloroflexi).

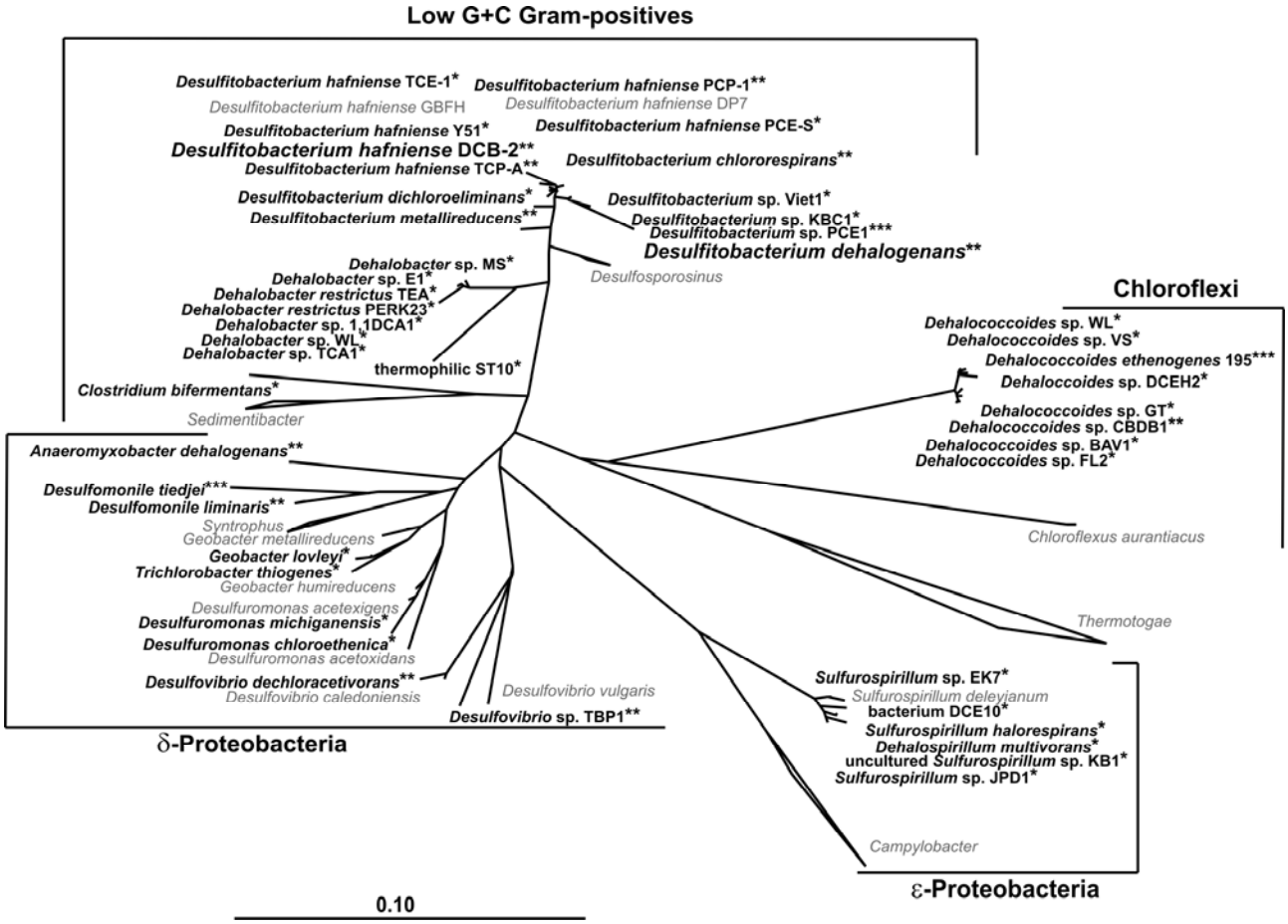
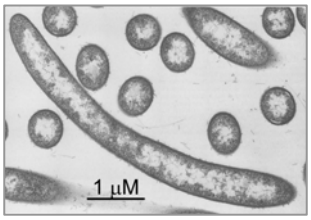
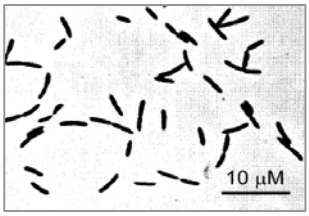


Figure 1.1 Phylogenetic tree of halorespiring bacteria (in bold) based on their 16S rRNA sequences. Dehalogenating capacities of the bacteria are indicated as: (*) chlorinated aliphatic compounds, (**) chlorinated aromatic compounds, and (***) both chlorinated aromatic and aliphatic compounds. The reference bar indicates 10 nucleotide exchanges per 100 nucleotides. By courtesy of H. Smidt.

They also share a unique feature, together with *Dehalobacter* spp., in their highly specialized halorespiring lifestyle being strictly dependent on the terminal reduction of chlorinated compounds coupled to hydrogen as the sole electron donor for their growth (Van de Pas, 2000; Van Eekert *et al.*, 2001). Other halorespiring bacteria that belong to the genera *Desulfitobacterium*, *Sulfurospirillum*, *Desulfomonile*, *Desulfuromonas*, *Desulfovibrio,* *Anaeromyxobacter*, *Trichlorobacter* and *Geobacter* show high metabolic diversity regarding the electron donors they can oxidize (e.g. formate, lactate, pyruvate, acetate, butyrate, fumarate) and the range of alternative electron acceptors that could enable the bacteria to grow under sulphate-, iron- or nitrate-reducing conditions besides halorespiration (Smidt & de Vos, 2004; Sung *et al.*, 2006).

Among halorespirers, the genus *Desulfitobacterium* (phylum *Firmicutes*, class *Clostridia*) includes a major group of isolates, consisting of more than five species that have the ability to degrade a wide range of chlorinated aliphatic and aromatic compounds. *Desulfitobacterium dehalogenans* JW/IU-DC1 was the first member of the genus, and was isolated from a methanogenic lake sediment (Utkin *et al.*, 1994), while *Desulfitobacterium hafniense* DCB-2 was obtained from municipal sludge (Christiansen *et al.*, 1996; Madsen *et al.*, 1992). Although these species are phylogenetically closely related (97.4% similarity of the 16S rRNA gene), important differences can be observed in the spectrum of organohalides they can dehalogenate and the specificity towards the position of the halogen substitution: *D. dehalogenans* JW/IU-DC1 is only able to reductively *ortho*-dechlorinate aromatic compounds, while *D. hafniense* DCB-2 is capable of *meta* dechlorination as well (Table 1.3).

Table 1.3 Growth properties of two type-strains from the genus *Desulfitobacterium*

	Fermentative	**Electron donors**	**Electron acceptors**	
			alternative	**halogenated** [g]
***Desulfitobacterium hafniense* DCB-2** [a-c] 	pyruvate, *DL*-tryptophane	pyruvate, lactate, formate [h], fumarate, butyrate [h], succinate, malate, ethanol, phenyl methyl ethers [j]	sulphite, thiosulphate, sulphur, nitrate, fumarate, Fe^{III}, Se^{VI}, Mn^{IV}, As^{V}	CHPA, 2,4-DCP, 3,5-DCP [i], 2,4,5-TCP, 2,4,6-TCP, PCP
***Desulfitobacterium dehalogenans* JW/IU-DC1** [c-f] 	pyruvate	pyruvate, lactate, formate, hydrogen	sulphite, thiosulphate, sulphur, nitrate, fumarate, Fe^{III}, Se^{VI}, Mn^{IV}, AQDS [k]	CHPA, 2,3-DCP, 2,4-DCP, 2,6-DCP, 2,3,4-TCP, 2,3,6-TCP, 2,4,6-TCP, PCP, 2,3,4,5-TeCP, 2,3,5,6-TeCP, 2,3,4,6-TeCP, *para*-hydroxylated PCBs

[a] (Madsen *et al.*, 1992); [b] (Christiansen *et al.*, 1996); [c] (Niggemyer *et al.*, 2001); [d] (Utkin *et al.*, 1994); [e] (Utkin *et al.*, 1995); [f] (Wiegel *et al.*, 1999); [g] *ortho* dechlorination activity; [h] contradicting results reported by (Christiansen *et al.*, 1996) and (Niggemyer *et al.*, 2001); [i] *meta* dechlorination activity; [j] (Neumann *et al.*, 2004); [k] (Luijten *et al.*, 2004b). Abbreviations: CHPA, 3-chloro-4-hydroxyphenylacetic acid; DCP, dichlorophenol; TCP, trichlorophenol; TeCP, tetrachlorophenol; PCP, pentachlorophenol; PCBs, polychlorinated biphenyls; AQDS, anthraquinone-2,6-disulfonate (humic acid analogue).

Catalysts of halorespiration

Reductive dehalogenases

The key step in halorespiration is the reductive removal of the halogen atom from the terminal electron acceptor, catalysed by a novel class of enzymes, the reductive dehalogenases. There are two major classes of reductive dehalogenases: the heme-containing 3-chlorobenzoate reductive dehalogenase from *D. tiedjei* (Ni *et al.*, 1995), and the vitamin B_{12}-dependent enzymes. Most of the B_{12}-dependent reductive dehalogenases also contain iron-sulphur (Fe-S) clusters and they are either embedded in or attached to the membrane by a small anchoring protein (Banerjee *et al.*, 2003; Smidt & de Vos, 2004). During the last 10 years, a number of reductive dehalogenases have been isolated and characterised from halorespiring organisms (Table 1.4), including the *ortho*-chlorophenol reductive dehalogenase (CprA) from *D. dehalogenans* (Van de Pas *et al.*, 1999), and the 3-chloro-4-hydroxyphenylacetate reductive dehalogenase from *D. hafniense* (Christiansen *et al.*, 1998). A common feature of the reductive dehalogenases is the presence of an amino-terminal Tat (twin-arginine-translocation) signal peptide which contains the conserved S/TRRXFLK sequence conferring them the capacity to be translocated through the cell membrane. The fundamental difference of the Tat protein translocation system from the general secretory (Sec) transport system is that proteins are transported across the cytoplasmic membrane in their folded form (Berks *et al.*, 2000). Proteins with the twin-arginine signal sequence often bind complex cofactors, such as iron-sulphur clusters, molybdopterin guanine dinucleotide or molybdopterin (Palmer *et al.*, 2005). It is assumed that the maturation of these complex cofactor-containing enzymes proceeds with the help of several assisting proteins. Indeed, reductive dehalogenase-encoding genes are often linked with genes whose products show high similarity to molecular chaperons and trigger factors (Villemur *et al.*, 2002). These proteins are involved in the process of protein maturation: molecular chaperons can prevent aggregation of the newly synthesized nascent polypeptides and can catalyze their correct folding in an ATP-dependent manner, while trigger factors enhance the rate of proline *cis/trans* isomerization, a rate limiting step in protein folding (Fink, 1999). Recent findings for the twin-argining signal peptide containing DMSO reductase (subunit DmsA) and the hydrogenase-1 (small subunit HyaA) from *E. coli* have demonstrated that signal peptides and chaperone proteins indeed operate in tandem to ensure coordinated assembly and export of their cognate substrate Tat-proteins (Jack *et al.*, 2004; Oresnik *et al.*, 2001).

Reaction mechanism

The reaction mechanism that underlies the dehalogenation reaction catalyzed by the iron-sulphur and B_{12}-containing reductive dehalogenases is still not fully understood. Experimental evidences support two models that describe the possible involvement of these cofactors in the electron transfer from a membrane-bound protein of the electron transport-chain to the halogenated hydrocarbon (Neumann *et al.*, 1996; Schumacher *et al.*, 1997).

Table 1.4 Properties of characterised reductive dehalogenases

Enzyme	*ortho*-CP reductive dehalogenase	CHPA reductive dehalogenase	2,4,6-TCP reductive dehalogenase	3,5-DCP reductive dehalogenase	3-CB reductive dehalogenase	CHBA reductive dehalogenase	CHPA reductive dehalogenase	PCE reductive dehalogenase
Organism	*Desulfitobacterium dehalogenans*	*Desulfitobacterium hafniense* DCB-2	*Desulfitobacterium hafniense* PCP-1	*Desulfitobacterium hafniense* PCP-1	*Desulfomonile tiedjei*	*Desulfitobacterium chlororespirans*	*Desulfitobacterium* sp. strain PCE1	
Gene	*cprA*	*cprA1*	*crdA*	*cprA5*	n.d.	*cprA*	n.d.	n.d.
Size	monomer 48 kDa	monomer 47 kDa	monomer (3 isoforms) 37 kDa	monomer 57 kDa	heterodimer 64 & 37 kDa	n.d. 50 kDa	n.d. 48 kDa	n.d. 48 kDa
Substrates	CHPA[o], 2-Br-4-CP[o], 2,3-DCP[o], 2,4-DCP[o]	CHPA[o]	2,4,6-TCP[o], PCP[o] 2,3,4,5-TeCP[o]	3,5-DCP[m], 2,3,5-TCP[m], 2,3,4,5-TeCP[p]	3-CB[m]	CHBA[o], CHPA[o], 2,3-DCP[o], 2,6-DCP[o], 2,4,6-TCP[o], hydroxy-PCB[o]	CHPA	PCE
Localization	membrane	membrane	membrane	membrane	membrane	membrane	membrane	membrane
Inducers	CHPA	CHPA	2,4,6-TCP	3,5-DCP	3-CB	CHBA	CHPA	PCE
Inhibitors	n.d.	n.d. [b]	sulfite, azide, nitrate	1-iodopropane, sulfite, cyanide, azide	n.d.	1-iodopropane, sulfite, cyanide, EDTA	1-iodopropane, N_2O, SO_3^{2-} [r]	1-iodopropane, N_2O, SO_3^{2-} [r]
Optimal temperature	52ºC	n.d.	n.d.	50ºC	38ºC	59ºC	n.d.	n.d.
Optimal pH	8.1	n.d.	7.0	6.8 – 7.0	7.2	6.8	n.d.	n.d.
Specific activity in cell extract [a]	308	540	112	31.5	0.15	85	30	20
Specific activity of purified protein [a]	28000	6180	380	400	18.5	15 400	4090	5510
Cofactors	1 cobalamin [c,d] 1 [3Fe-4S] [d] 1 [4Fe-4S] [d]	1 cobalamin [d] 12Fe-13S	1 cobalamin [c,d]	1 cobalamin [c] Fe-S[e]	Heme Fe-S [d]	1 cobalamin [c,d] 2 [4Fe-4S]	1 cobalamin [c] n.d.	1 cobalamin [c] n.d.
O_2-sensitivity ($t_{1/2}$)	≅ 90 min	n.d.	sensitive[f]	110 min	n.d.	77 min	n.d.	n.d.
References	(Van de Pas *et al.*, 1999)	(Christiansen *et al.*, 1998)	(Boyer *et al.*, 2003)	(Thibodeau *et al.*, 2004)	(Ni *et al.*, 1995)	(Krasotkina *et al.*, 2001; Loffler *et al.*, 1996)	(Van de Pas, 2000; Van de Pas *et al.*, 2001)	

Enzyme	PCE/TCE reductive dehalogenase	PCE/TCE reductive dehalogenase	PCE/TCE reductive dehalogenase	TCE reductive dehalogenase	PCE reductive dehalogenase	PCE/TCE reductive dehalogenase	PCE/TCE reductive dehalogenase
Organism	*Clostridium bifermentans* DPH-1	*Desulfitobacterium hafniense* PCE-S	*Dehalobacter restrictus*	*Dehalococcoides ethenogenes* strain 195		*Dehalospirillum mutivorans*	*Desulfitobacterium hafniense* Y51
Gene	*pceC*	*pceA*	*pceA*	*tceA*	*pceA*	*pceA*	*pceA*
Size	homodimer 35 kDa per monomer	homotrimer [g] 65 kDa per monomer	monomer 60 kDa	monomer 61 kDa	monomer 51 kDa	monomer 58 kDa	monomer 58 kDa
Substrates	PCE, TCE, *cis/trans*-DCE	PCE, TCE	PCE, TCE, chlorethanes, tetrachloromethane	TCE, *cis/trans*-DCE, VC	PCE	PCE, TCE, chlorinated propenes	PCE, TCE, chloroethanes
Localization	peripheral membrane	membrane	membrane	membrane	membrane	cytoplasm [h]	periplasmic fraction
Inducers	n.d.	constitutive	n.d.	n.d.	constitutive	PCE [i]	PCE, TCE
Inhibitors	1-iodopropane, titanium citrate	sulfite, cyanide, azide, EDTA [r]	1-iodopropane, cyanide	1-iodopropane, sulfite, cyanide, azide, dithionite	1-iodoethane, sulfite, cyanide, azide, dithionite	1-iodopropane, sulfite, cyanide, EDTA, chloro-methanes, nitrite	1-iodopropane, sulfite
Optimal temperature	35ºC	50ºC	n.d.	n.d.	n.d.	42ºC	37ºC
Optimal pH	7.5	7.2	8.1	n.d.	n.d.	8.0	7.0 – 7.5
Specific activity in cell extract [a]	6.5 [j]	240	610	510	270	25	45 [k]
Specific activity of purified protein [a]	59.5 [j]	39 000	13 600	12 000	20 500	2640	114 [k]
Cofactors	1 cobalamin [c]	1 cobalamin [d] 8Fe/8S [d]	1 cobalamin [c,d] 2 [4Fe-4S] [d]	1 cobalamin [c] Fe-S	1 cobalamin [c] Fe-S	1 cobalamin [c] 8Fe/8S [d]	1 cobalamin [c] Fe-S
O_2-sensitivity ($t_{1/2}$)	20 h	50 min	280 min	n.d.	n.d.	120 min	330 min
References	(Okeke *et al.*, 2001)	(Miller *et al.*, 1997; Miller *et al.*, 1998)	(Maillard *et al.*, 2003; Schumacher *et al.*, 1997)	(Magnuson *et al.*, 2000; Magnuson *et al.*, 1998)	(Magnuson *et al.*, 1998), (Morris *et al.*, 2006)	(Neumann *et al.*, 2002; Neumann *et al.*, 1996; Neumann *et al.*, 1998)	(Suyama *et al.*, 2002)

[a] One unit (U) of enzymatic activity is defined as 1 nmol chloride released or 2 nmol methylviologen oxidized × min^{-1} × mg $protein^{-1}$; [b] Inhibition by 1-iodopropane was not detected ; [c] Indicated by photoreversible inactivation of the reduced enzyme by iodoalkanes; [d] Indicated by spectroscopic analysis; [e] Ferredoxin-type conserved motif in encoding gene; [f] 70% activity decrease in the presence of 10% air in 1h; [g] Presumably most of the molecular mass is contributed by tightly associated detergent (Krasotkina *et al.*, 2001); [h] Catalytically active subunit; [i] Also constitutive and non-inducible strain isolated; [j] One unit was defined as 1 nmol TCE and *cis*-DCE produced under assay conditions; [k] One unit was defined as 1 nmol PCE dechlorinated × min^{-1}; [m] Dehalogenation at the *meta*-position; [o] Dehalogenation at the *ortho*-position; [p] Dehalogenation at the *para*-position; [r] in cell extracts. ABBREVIATIONS: CB, chlorobenzoate; CP, chlorophenol; DCP, dichlorophenol; TCP, trichlorophenol; TeCP, tetrachlorophenol; CHBA, 3-chloro-4-hydroxybenzoate; CHPA, 3-chloro-4-hydroxyphenylacetic acid; PCB, polychlorinated biphenyl; PCE, tetrachloroethene; TCE, trichloroethene; DCE, dihloroethene VC, vinylchloride; n.d., not determined.

One model presumes that an organocobalt adduct is formed, like in methyltransferases (Fig. 1.2a); according to the second model, however, a radical intermediate is involved in the dechlorination reaction (Fig. 1.2b), as has been described for B_{12}-dependent ribonucelotide reductases (Banerjee *et al.*, 2003). To shed light on the reaction mechanism of reductive dehalogenases, further studies on the wild-type and mutant enzymes obtained by directed mutagenesis will be required.

Transcriptional regulation of halorespiration

Induction of the dehalogenation pathway

Growth experiments in the absence or presence of various chlorinated compounds showed that haloaromatic reductive dehalogenase activity was only detectable under halorespiring conditions, which suggested a strong allosteric or transcriptional control of these enzymes. When *D. dehalogenans* cells were cultivated in the presence of chlorinated compounds and sublethal concentrations of chloramphenicol, an inhibitor of *de novo* protein synthesis, dechlorination activity was not detected (Utkin *et al.*, 1995). This was the first evidence that halorespiration is controlled at the gene expression level. Interestingly, chlorinated compounds that were degraded by *D. dehalogenans* clustered in two groups: compounds with substitution at positions 2 and 4 (in respect to the hydroxyl group at position one) were able to induce dehalogenation activity, while those with an additional substitution at position 6 were non-inducers, despite the fact that *D. dehalogenans* was able to dechlorinate them at the *ortho* position.

Further details were revealed on the transcriptional control of halorespiration genes by Northern blot analysis of RNA obtained from non-halorespiring and from 3-chloro-4-hydroxypahenylacetic acid (CHPA) grown *D. dehalogenans* cells (Smidt *et al.*, 2000b). The chlorophenol reductive dehalogenase (*cpr*) gene cluster that is responsible for CHPA degradation consists of eight genes (*cprTKZEBACD*), among which *cprA* codes for the *ortho*-chlorophenol reductive dehalogenase (Van de Pas *et al.*, 1999). Transcription was initiated from three promoters in the presence of CHPA, resulting in *cprT* and *cprZE*, *cprBA* or *cprBACD* polycistronic transcripts (Smidt *et al.*, 2000b). Remarkably, it was shown that transcription of the *cprBA* genes increased 15-fold after 30 minutes of CHPA addition, demonstrating control at the level of transcriptional initiation (Smidt *et al.*, 2000b). Similar control of the PCE-degrading *prdAB* operon was observed in *Desulfitobacterium* sp. strain KBC1 (Tsukagoshi *et al.*, 2005). In the *cpr* operon of *D. dehalogenans*, only the putative CRP/FNR-type transcriptional regulator encoding *cprK* gene was constitutively expressed under pyruvate-fermenting, and nitrate-, fumarate-, or halorespiring conditions. Further studies revealed that CprK directly interacts with CHPA, which causes an allosteric change in the regulator and enables it to bind to an inverted repeat (dehalobox) in the promoter region of the *cprBA* operon and initiate transcription (Pop *et al.*, 2004). It was also shown that CprK is subject to reversible oxidative inactivation, which would enable the bacteria to down-regulate CprK-mediated induction of gene expression in the presence of CHPA when the cells are under oxidative stress conditions.

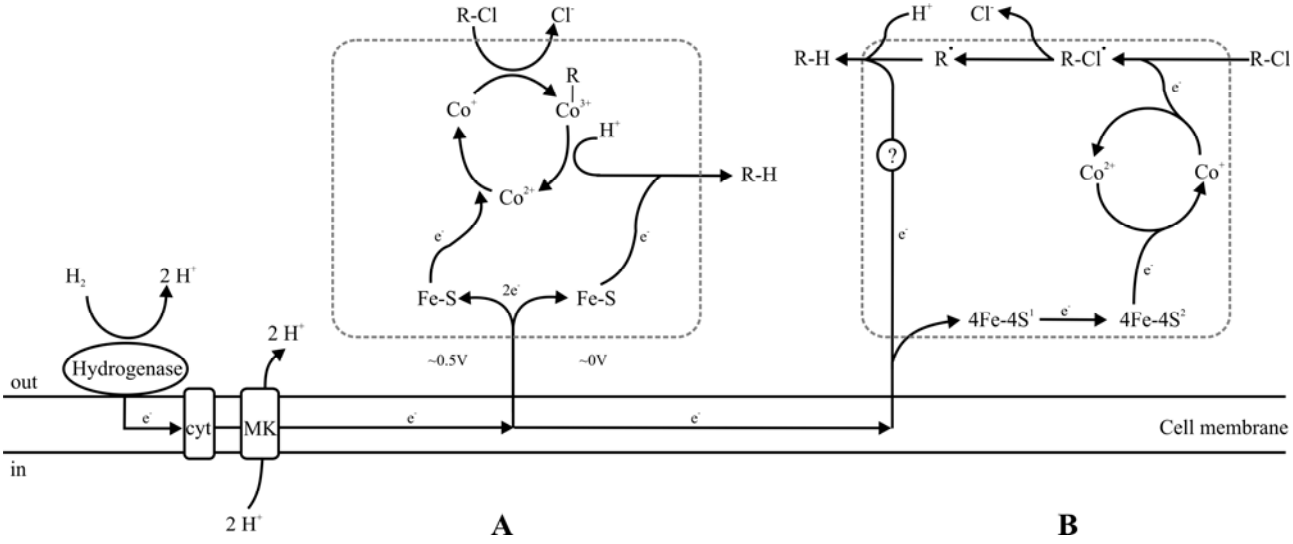


Figure 1.2 Proposed reaction mechanisms of reductive dehalogenases (after Middeldorp *et al.*, 1999). (**A)** Covalent halocarbon intermediate formation. (**B**) Radical formation. Abbreviations: cyt, cytochrome; MK, menaquinone.

In vitro experiments provided the evidence for the formation of two disulphide bridges in CprK upon oxidation and suggested that the presence of the disulphide bridges does not change affinity of the protein to the effector compound CHPA, but affects its DNA-binding capacity, probably by fixing the DNA-binding domains in an inactive conformation (Pop *et al.*, 2006). The physiological relevance of such redox regulation of CprK in *D. dehalogenans* is still under investigation.

In the closely related *D. hafniense* DCB-2, a highly similar *ortho*-chlorophenol reductive dehalogenase gene cluster was identified consisting of *cprTK$_1$ZEBA$_1$C* open reading frames (Villemur *et al.*, 2002). The reductive dehalogenase CprA1 has been characterised and found to be able to catalyze the *ortho*-dehalogenation of CHPA (Christiansen *et al.*, 1998). Similarly to CprA from *D. dehalogenans*, the protein was only produced under halorespiring conditions and not when the cells are grown with fumarate as electron acceptor, suggesting a strong induction of the *cprA1* gene by chlorinated compounds (Christiansen *et al.*, 1998). CprK1, a protein that is encoded in the same *cpr* gene cluster as CprA1 and shows high homology to CprK from *D. dehalogenans* (89% protein sequence identity), is a potential regulator that may control transcriptional initiation in the *cpr* gene cluster of *D. hafniense* under halorespiring conditions.

CRP-FNR family of transcriptional regulators

The first transcriptional regulators that were identified and gave also name to the family, which now comprises over 350 members, were the cAMP receptor protein (CRP) also known as catabolite gene activator protein (CAP), and the fumarate and nitrate reduction regulator (FNR) of *Escherichia coli* (Green *et al.*, 2001).

The 24-kDa CRP protein senses cAMP, a molecule whose level is dependent on the cell physiology and is elevated under glucose starvation conditions (Busby *et al.*, 1996; Kolb *et al.*, 1993). The effector-bound CRP:cAMP complex interacts with a specific DNA sequence (CRP-box, TGTGA-N_6-TCACA) with high affinity (Harman, 2001). CRP can bind to target promoters at two distinct positions: at position -61.5 (class I) or at position -41.5 (class II) relative to the transcription start site. At class I CRP-dependent promoters, transcription activation involves protein-protein interactions between CRP and the RNA polymerase (RNAP) α-subunit C-terminal domain that facilitate binding of RNAP to promoter DNA to form the RNAP-promoter closed complex. At class II CRP-dependent promoters, since CRP overlaps the -35 region which is necessary for the binding of RNAP, transcription activation involves both: (i) the previously described interactions between CRP and RNAP α-subunit C-terminal domain; and (ii) protein-protein interactions between CRP and RNAP α-subunit N-terminal domain that facilitates isomerization of the RNAP-promoter closed complex to the RNAP-promoter open complex (Busby *et al.*, 1999; Savery *et al.*, 1996). It has been recently shown that CRP makes also contact with the σ^{70}-factor of the RNA polymerase at class II promoters, although the predominant interaction still occurs between CRP and the RNAP α-subunit (Rhodius *et al.*, 2000).

The number of genes that are controlled by the same regulator in *E. coli* is remarkably large: over 100 genes are under the control of CRP, while more than 120 genes that are involved in anaerobic respiration of e.g. fumarate, nitrate, nitrite, DMSO, are regulated by FNR (Bauer *et al.*, 1999; Harman, 2001). FNR is a 30 kDa-protein which binds to inverted repeats (FNR-box, TTGAT-N_4-ATCAA) in its target promoters under anaerobic conditions (Guest *et al.*, 1996; Spiro, 1994; Spiro *et al.*, 1990). The most notable feature of FNR, which distinguishes it from CRP is the presence of four cysteine residues in the protein that are involved in the coordination of an [4Fe-4S] cluster (Green *et al.*, 1993). This redox sensitive prosthetic group can reversibly be oxidized into [2Fe-2S] clusters, which results in the formation of an inactive apo-FNR (Bauer *et al.*, 1999). The presence of the intact [4S-4S] cluster possibly increases dimerization of FNR with a yet-unknown mechanism (Kiley *et al.*, 1998). FNR, similarly to CRP, generally binds its target promoters at conserved distance from the transcription start site (class I and II promoters). It makes contact with the C-terminal domain of the RNAP α-subunit (both class I and class II promoters), and predominantly with the σ^{70}-factor of the RNAP (class II promoters), although recently a contact with the N-terminal domain of the RNAP α-subunit has also been demonstrated (Blake *et al.*, 2002; Green *et al.*, 2001).

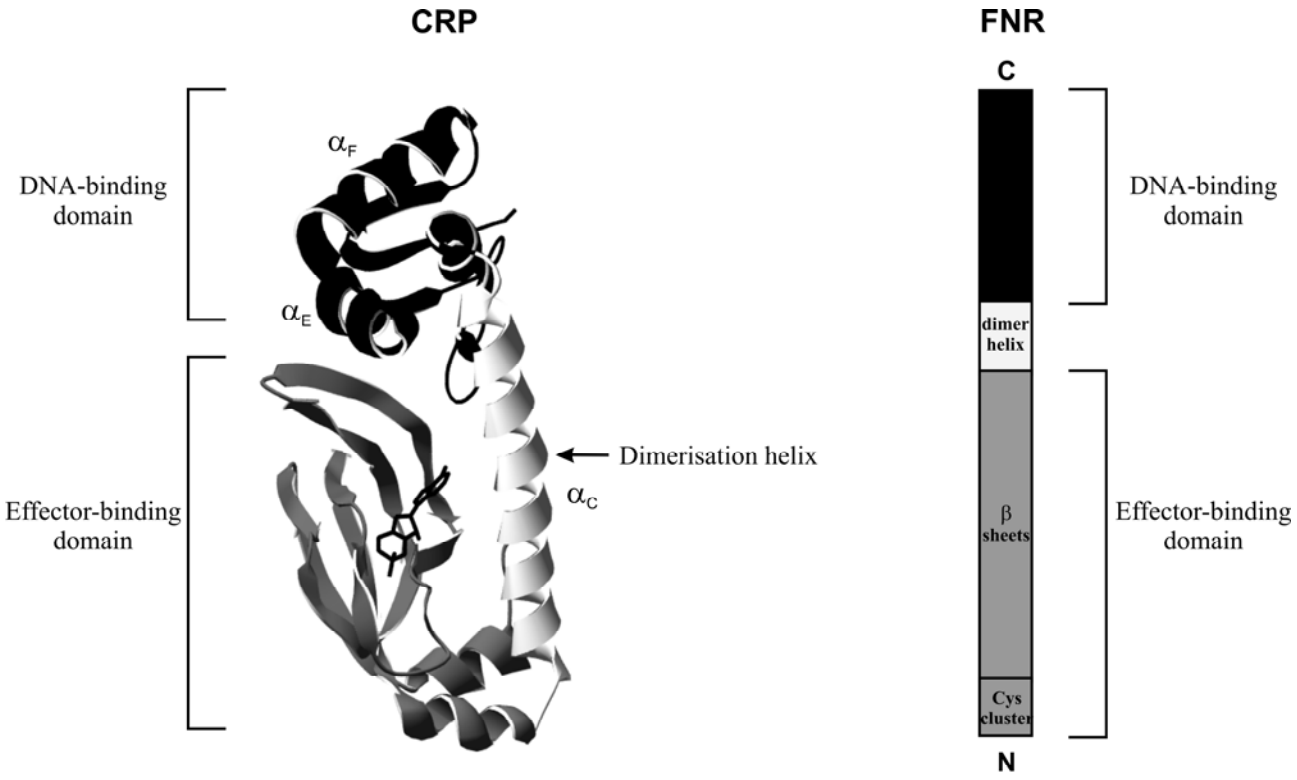


Figure 1.3 Secondary structures of CRP and FNR. The DNA-binding domains are coloured black, with the characteristic helix-turn-helix motif (α_E and α_F) highlighted in CRP, in light grey are the long dimerisation helixes (α_C in CRP) and in dark grey is the effector-binding β-barrel domain. The N-terminal extension in FNR contains conserved cysteine residues involved in [4Fe-4S] binding. The CRP structure is based on PDB 1I5Z, with the bound cAMP molecule (sticks) shown in the effector-binding domain.

Structural basis of transcriptional activation

Proteins from the CRP-FNR family form homodimers and share a common basic scaffold consisting of three distinct domains (Fig. 1.3). The N-terminal sensory domain that contains the ligand or allosteric cofactor binding site is connected to the C-terminal helix-turn-helix (HTH) DNA-binding domain through a long dimerization α-helix (Chan, 2000). The sensory domains evolved to perceive different signal molecules in the different family members thus enabling them to control the expression of a wide range of metabolic genes, including those involved in nitrogen fixation, denitrification, arginine catabolism, aromatics degradation, pathogenesis or CO oxidation (Korner *et al.*, 2003). Crystallization trials aimed at answering two questions: first, what are the structural effects of the binding of an effector molecule; and second, how this signal is transmitted from the sensory domain to the DNA-binding domains.

CRP was the first protein from the family whose secondary structure was resolved in the presence of the effector molecule cAMP (on-state) (McKay *et al.*, 1982). Next, the determination of the 3D-structure of the cAMP-bound CRP in complex with its target DNA revealed an interesting feature of the protein (Schultz *et al.*, 1991). CRP induced a 90° bend in the DNA, originating from two kinks that occurred in the conserved TGTGA-N_6-TCACA sequence at symmetry related positions. The proposed effect of DNA bending on transcriptional activation was (i) promoting the proper orientation of CRP and RNAP for direct interaction, and (ii) enabling a contact between the RNAP and DNA upstream of the CRP binding site.

Finally, the recently solved structure of CRP in complex with its target DNA and the RNAP α-subunit C-terminal domain (αCTD) showed that the protein-protein interaction interface is relatively small and does not promote large conformational changes in CRP and the αCTD, which supports the simple "recruitment" theory of transcriptional activation (Benoff *et al.*, 2002). Despite of numerous trials, the crystal structure of the effector-free (off-state) CRP, which would provide us with essential details on the allosteric effect of signal molecule binding, has not yet been solved.

The first off-state structure of a CRP/FNR-type protein was obtained from *Rhodospirillum rubrum*, a photothrophic α-proteobacterium that grows anaerobically in the presence of CO. The transcriptional regulator, CooA senses CO via a heme group (He *et al.*, 1999). Superimposition of the off-state CooA structure with that of the on-state CRP (root mean square deviation was 1.16 Å for the sensory domains) revealed a few important differences (Lanzilotta *et al.*, 2000). First of all, the DNA-binding domain adopts a strikingly different position in CooA that hinders binding to its target DNA in the effector-free state. Also, structural comparisons revealed that binding of cAMP in CRP induces movement of a hairpin loop (β_4-β_5 in CRP) and causes the long dimerisation helix (α_C in CRP), which connects the sensory domain with the DNA-binding domain, to bend around a hinge region (Fig. 1.4).

Although the exact mechanism of the effector-dependent repositioning of the DNA-binding domains is not known, Lanzilotta *et al*. (2000) suggest a critical role of the above mentioned conserved hinge region and the hairpin loop (Chan, 2000). This theory is supported by additional evidence based on the recently solved crystal structures of the CRP/FNR-type transcriptional regulator of pathogenicity from *Listeria monocytogenes*, PrfA and its constitutively active mutant $PrfA_{G145S}$ (Eiting *et al.*, 2005). These structural data showed that mutation at position 145 in the conserved hinge region fixed the protein in the constantly active DNA-binding state, providing further support for the involvement of this region in transmitting signal from the sensory domain to the DNA-binding domain. Also, the hairpin loop sequence is highly conserved in many CRP/FNR-type proteins, including CooA, PrfA and the halorespiration regulator CprK proteins (Fig. 1.4).

Besides crystallographic studies, a considerably new technique, the electrospray ionisation-coupled mass spectrometry (μESI-MS) (reviewed by Van den Heuvel *et al.*, 2004), also in combination with limited proteolysis, is a promising tool for the analysis of non-covalent protein-DNA interactions which might enable us to get more insight on the ligand-induced conformational changes and dynamics of CRP/FNR-type transcriptional regulators.

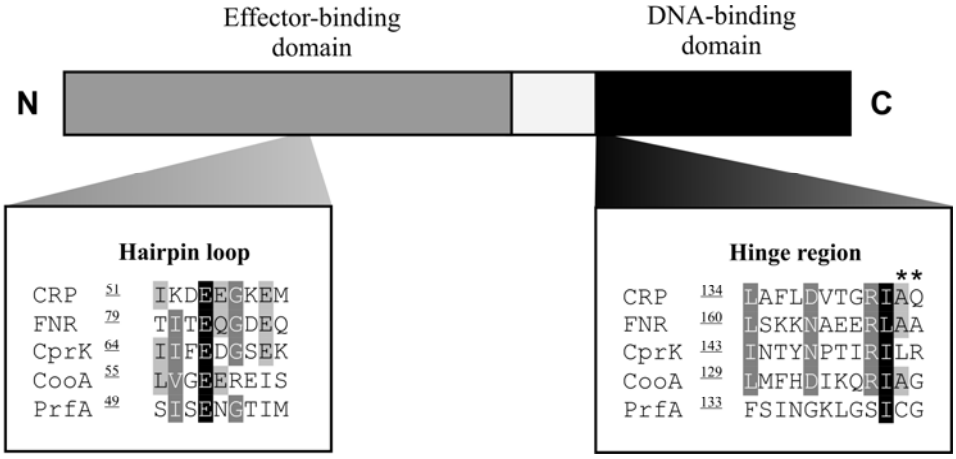


Figure 1.4 Sequence alignment of the hairpin loop and hinge regions involved in transmitting the effector binding signal from the sensory domain to the DNA-binding domain in CRP/FNR-type transcriptional regulators. Stars indicate the position where mutations resulted in a constitutive DNA-binding conformation of the corresponding regulator: A145T in CRP and G145S in PrfA.

Complexity of transcriptional regulation

Studies on the expression of reductive dehalogenase genes that code for the catalytic enzymes of halorespiration have revealed unexpected complexity. The 2,4,6-TCP reductive dehalogenase encoding *crdA* is constitutively expressed in *Desulfitobacterium hafniense* strain PCP-1, regardless of the absence or the presence and type of chlorinated compound (PCE, PCP, 3,5-DCP) (Gauthier *et al.*, 2006). However, CrdA enzymatic activity is only measured in cell-extracts that were obtained from 2,4,6-TCP-grown cultures (Boyer *et al.*, 2003). Similarly, *cprA5* that encodes a *meta*-chlorophenol reductive dehalogenase in strain PCP-1 is induced not only by its substrate 3,5-DCP but also by 2,4,6-TCP, although enzymatic activity can only be measured when the growth medium was amended with 3,5-DCP (Gauthier *et al.*, 2006). These may indicate that other crucial elements, such as the synthesis of reductive dehalogenase-specific corrinoid cofactors (Siebert *et al.*, 2002), or the production of halorespiration-specific electron transport proteins, such as a cytochrome-*c* in *Desulfomonile tiedjei* (Louie *et al.*, 1997), are needed to be co-induced with the reductive dehalogenase genes to achieve dehalogenation activity in the cells. Studies are awaiting that will address the apparent complexity of the control of the halorespiration machinery, which might reveal the presence of more than one, synergistic or hierarchical regulatory mechanisms.

Our concepts on transcriptional regulation of halorespiration can be further fine tuned by data obtained from growth studies on the availability of alternative electron acceptors other than halogenated hydrocarbons (McCarty, 1997).

Early studies with enrichment and pure dehalogenating cultures concluded that oxygen, nitrate, and sulphate generally inhibit dehalogenation but the nature of such inhibition varied under different biological and chemical conditions (Mohn *et al.*, 1992). Luijten *et al*. have found that in the presence of lactate (29 mM) as electron donor, nitrate (10 mM) was preferentially used over PCE (10 mM) in *Sulfurospirillum halorespirans* (Luijten, 2004). When they examined the role of the availability of electron donors on halorespiration, it was found that dehalogenation of PCE to *cis*-1,2-DCE could occur at H_2 concentration as low as 0.5 nM (Luijten *et al.*, 2004a). This concentration is much lower than what is needed for bacterial methanogenesis (5-100 nM H_2) or for sulphate reduction (1-10 nM), but is in similar range with H_2-threshold values for iron and nitrate reduction (0.1-0.8 and <0.05 nM, respectively). The influence of alternative electron acceptors on PCE and TCE degradation was also investigated in *D. hafniense* strain TCE1 (Gerritse *et al.*, 1999). The presence of excess lactate (40 mM) as electron donor allowed for dechlorination of PCE even in the presence of the mixture of nitrate, fumarate and sulphite (2 mM each). However, under lactate-limiting conditions (10 mM lactate), and in the presence of excess electron acceptors (10 mM nitrate, 10 mM fumarate, 10 mM sulphite), halorespiration on PCE was not carried out. These results indicate that the relative availability of electron donors and acceptors in the environment is more important for predicting halorespiration capacity than the actual concentrations of competitive electron donors (Gerritse, *et al.* 1999). In *Escherichia coli*, the ATP yield is thought to be the most important regulatory criterion where electron acceptors of higher priority repress pathways of lower priority (Unden *et al.*, 1994). Indeed, priority categories based on the Gibbs free energy changes correlate well with the actual physiological preference of the bacteria towards various electron acceptors: first oxygen is used (ΔG_0' = -2830 kJ/mol glucose), then nitrate (ΔG_0' = -858 kJ/mol), followed by DMSO, TMAO and fumarate ((ΔG_0' = -550 to -650 kJ/mol). The underlying principle in transcriptional control is most probably very complex, including bacterial promoters that contain multiple regulator binding sites, or global regulators (e.g. IHF, integration host factor) and regulatory compounds (as the alarmone (p)ppGpp) that can alter expression of genes according to the physiological state of the cell (Barnard *et al.*, 2004; Cases *et al.*, 2005; Diaz, 2004; Shingler, 2003).

Genomics of halorespiring bacteria

Very recently, the genome sequence of two halorespiring organisms, *Dehalococcoides ethenogenes* strain 195 (1.47 Mbp) and strain CBDB1 (1.4 Mbp) has been determined (Seshadri *et al.*, 2005) (Kube *et al.*, 2005). This revealed that despite their relatively small genome size, a surprisingly large number of putative reductive dehalogenases (RD) are encoded in their genome (17 and 32, respectively). The partially sequenced genome of another halorespiring organism, *Desulfitobacterium hafniense* DCB-2 (4.9 Mbp in 2001, www.jgi.doe.gov) contains also multiple copies of reductive dehalogenase-encoding genes (Villemur *et al.*, 2002). It is likely that the large number of RD genes has been obtained via the combination of gene duplication and horizontal gene transfer events.

The latter has been suggested to be the main cause for the presence of multiple RD genes in *D. ethenogenes* strain 195. These genes are located in "foreign", atypical regions of the chromosome, showing different Karlin signature from the rest of the genome (Regeard *et al.*, 2005). Furthermore, it also appears that RD genes are often associated with transposable elements and insertion sequences (Maillard *et al.*, 2005), which enables transmission of novel catabolic properties via horizontal gene transfer (Janssen *et al.*, 2005) (Smidt & de Vos, 2004). Besides being a robust and very efficient way of conferring novel capabilities upon the recipient organism, horizontal gene transfer is also proposed to be the major drive for the formation of operons in prokaryotes ("selfish operon" model) (Lawrence, 2003). This model states that functionally related genes tend to cluster in the chromosome to increase their chance to co-mobilize during horizontal gene transfer. It was also suggested that the presence of the responsible transcription factor-encoding gene in *cis* gives additional advantage when a gene cluster conferring new metabolic properties is transferred to the recipient. Indeed, the reductive dehalogenase-encoding genes in *D. ethenogenes* strains are surprisingly often linked with two-component sensory protein-encoding genes or with MarR-type transcriptional regulators (Kube *et al.*, 2005). Similarly, more *cprK* genes are present in *D. hafniense* DCB-2 that code for putative transcriptional regulators from the CRP-FNR family and cluster with reductive dehalogenase-encoding genes (Gábor *et al.*, 2006).

The latest release of completed microbial genomes contains the DNA sequence of several environmental bacteria that are capable of degrading a wide variety of pollutants, including the PCE-degrading halorespirer *D. hafniense* Y51 (Galperin, 2006). The 5.7 Mbp genome contains only two RD genes, but an unprecedented variety of genes that enable the organism to use alternative electron acceptors (DMSO, fumarate, nitrate, nitrite, sulphite and possibly sulphate, besides metal ions) in combination with a variety of electron donors (pyruvate, lactate, formate and phenyl-methyl ethers) (Nonaka *et al.*, 2006). The genome of *D. hafniense* strain Y51 contains the complete pathway for cobalamin synthesis (essential cofactor for reductive dehalogenases), in contrast to *Dehalococcoides* species, that are not capable of *de novo* cobalamine synthesis, and rather obtain this cofactor by uptake and salvage from the environment. The available sequence data of *D. hafniense* DCB-2 suggests also that this strain can synthesize cobalamine (at least 17 of 20 genes are present). Another main difference between the dechlorination specialist *Dehalococcoides* and the generalist *Desulfitobacterium* species is that the genome of the coccoid organism encodes no motility, while both *Desulfitobacterium* species, known to possess flagelli, encode a large number of motility genes and multiple copies of methyl-accepting chemotaxis genes which might enable these bacteria to actively seek for chlorinated hydrocarbons. Bacterial chemotaxis towards environmental pollutants has a significant role not only in locating an energy source for the cells, but in facilitating the transfer of mobile genetic elements and catabolic plasmids by directing motile bacteria to contaminated sites where strains carrying the relevant catabolic plasmids are likely to be present, thus likely to assist and enhance the rate of bioremediation (Parales *et al.*, 2002).

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Characterisation of CprK1, a CRP/FNR-type transcriptional regulator of halorespiration from *Desulfitobacterium hafniense*

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Abstract

The recently identified CprK branch of the CRP (cyclic AMP receptor protein)-FNR (fumarate and nitrate reduction regulator) family of transcriptional regulators includes proteins that activate the transcription of genes encoding proteins involved in reductive dehalogenation of chlorinated aromatic compounds. Here we report the characterisation of the CprK1 protein from *Desulfitobacterium hafniense*, an anaerobic low G+C gram-positive bacterium that is capable of reductive dechlorination of 3-chloro-4-hydroxyphenylacetic acid (CHPA). The gene encoding CprK1 was cloned and functionally overexpressed in *Escherichia coli*, and the protein was subsequently purified to homogeneity. To investigate the interaction of CprK1 with three of its predicted binding sequences (dehaloboxes), we performed *in vitro* DNA-binding assays (electrophoretic mobility shift assays) as well as *in vivo* promoter probe assays. Our results show that CprK1 binds its target dehaloboxes with high affinity (dissociation constant, 90 nM) in the presence of CHPA and that transcriptional initiation by CprK1 is influenced by deviations in the dehaloboxes from the consensus TTAAT----ATTAA sequence. A mutant CprK1 protein was created by a Val→Glu substitution at a conserved position in the recognition α-helix that gained FNR-type DNA-binding specificity, recognising TTGAT----ATCAA sequence (FNR box) instead of the dehaloboxes. CprK1 was subject to oxidative inactivation *in vitro,* most likely caused by the formation of an intermolecular disulfide bridge between Cys11 and Cys200. The possibility of redox regulation of CprK1 by thiol-disulfide exchange reaction was investigated by two Cys→Ser mutants. Our results indicate that a Cys11-Cys200 disulfide bridge does not appear to play a physiological role in the regulation of CprK1.

Introduction

Transcriptional regulators of the CRP-FNR family play an important role in modulating the expression of numerous metabolic genes in many facultative or strictly anaerobic bacteria. These structurally related regulators evolved to enable an efficient response to a wide range of environmental signals in organisms that are often phylogenetically distinct. In *Escherichia coli*, CRP is a positive regulator of catabolite repression by directly sensing elevated cAMP levels caused by glucose starvation and activating the expression of genes involved in the utilization of alternative carbon sources (Kolb *et al.*, 1993). *E. coli* FNR senses the changes in redox state (e.g. the decreasing availability of oxygen) through a polynuclear iron-sulphur centre, whose presence enhances DNA binding, resulting in transcriptional activation of genes involved in anaerobic respiration (Spiro *et al.*, 1990). Apart from these archetypes of the CRP-FNR superfamily, there are several members that use other effector molecules that can stimulate or repress the expression of metabolic genes by causing structural changes in the DNA-binding domain of the corresponding regulator (Harman, 2001; Korner *et al.*, 2003). Binding of carbon monoxide to CooA from *Rhodospirillum rubrum* is necessary for the ability to regulate the expression of genes for CO-oxidative growth (He *et al.*, 1999).

NtcA, a regulator of nitrogen-balance in *Anabaena* spp. is activated by 2-oxoglutarate (Tanigawa *et al.*, 2002), while the FNR-like protein (FLP) in *Lactococcus lactis* is activated by the formation of an intramolecular disulfide bridge between Cys5 and Cys102 caused by oxidative stress (Gostick *et al.*, 1998).

A branch of the CRP-FNR family that has recently emerged, CprK, includes transcriptional regulators that mediate the response to halogenated aromatic compounds. Halogenated hydrocarbons with a high degree of substitution are chemically very stable under anaerobic conditions, they are persistent and can cause toxic effects for a long period of time (WHO, 1989). Several recently discovered strictly anaerobic bacteria are capable of halorespiration, a novel respiratory pathway in which halogenated hydrocarbon compounds serve as terminal electron acceptors (El Fantroussi *et al.*, 1998). During halorespiration, one or more halogen atoms are reductively removed from the halogenated hydrocarbon while energy is conserved via electron transport-coupled phosphorylation. An important group among halorespiring bacteria consists of members of the genus *Desulfitobacterium,* which belong to the low G+C gram-positive bacteria and shows high metabolic versatility in its capability to degrade halogenated phenol derivatives. *Desulfitobacterium dehalogenans* can reductively dechlorinate halogenated phenolic compounds at the *ortho* position (Utkin *et al.*, 1994), while *Desulfitobacterium hafniense* DCB-2 can dechlorinate 3,5-dichlorophenol at the *meta* position in addition to *ortho*-substituted compounds (Christiansen *et al.*, 1996). The reductive dehalogenase CprA has been isolated from *D. dehalogenans*; it catalyses the dechlorination of 3-chloro-4-hydroxyphenylacetic acid (CHPA) at the *ortho* position (Van de Pas *et al.*, 1999). The corresponding *cprA* gene was isolated and found to be located in a *c*hloro*p*henol *r*eductive dehalogenase gene cluster containing *cprTKZEBACD* open reading frames (Smidt *et al.*, 2000). Transcription of the *cpr* gene cluster was initiated from three promoters under halorespiring conditions resulting in *cprT*, *cprZE* and *cprBA* or *cprBACD* transcripts, while *cprK* was constitutively transcribed at a basic level. The deduced amino acid sequence of the protein encoded by *cprK* showed significant homology to members of the CRP-FNR family, suggesting a possible role in transcriptional regulation of the *cpr* gene cluster. In addition, putative regulator binding sites were identified in the three promoter regions that resembled the FNR consensus TTGAT----ATCAA (Smidt *et al.*, 2000). Recently, biochemical studies have proved that CprK from *D. dehalogenans* is a transcriptional activator that binds CHPA with high affinity, resulting in an active DNA-binding conformation which enables CprK to bind to a specific DNA sequence, TTAATacgcACTAA, located in the promoter region of *cprBA* (Pop *et al.*, 2004). CprK was subject to inactivation by diamide, which converts free thiols to disulfides, suggesting a putative role of redox regulation in activation of CprK mediated by one or more of the five cysteines present in the protein.

The recently elucidated genome sequence of *D. hafniense* DCB-2 (www.jgi.doe.gov) revealed the presence of several *cprK* homologues (Smidt & de Vos, 2004). The gene *cprK1* is situated in a *cpr* gene cluster which contains $cprTK_1ZEBA_1C$ open reading frames, among which the product of the *cprA1* gene, a CHPA reductive dehalogenase has been isolated and characterised (Christiansen *et al.*, 1998).

This reductive dehalogenase was isolated from *D. hafniense* cells grown on pyruvate as electron donor and CHPA as electron acceptor. It was reported that, when the cells were grown in a medium containing fumarate instead of CHPA as electron acceptor, no dehalogenation activity could be detected, suggesting a strong induction of the corresponding *cprA1* gene by CHPA. In our study, we have functionally overexpressed the gene encoding CprK1, and purified the protein in order to study its interaction *in vivo* and *in vitro* with three putative target sequences (dehaloboxes) in the promoter region of *cprT*, *cprZ* and *cprB* genes. Next, we have investigated the DNA-protein contact in detail, using a CprK1 mutant with altered DNA-binding specificity. Finally, the question of possible redox regulation of this CprK-homologue was also addressed with the help of CprK1 Cys→Ser mutants.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Desulfitobacterium hafniense DCB-2 was grown in phosphate-bicarbonate buffered medium with a low chloride concentration (Holliger *et al.*, 1993) under N_2/CO_2 (80:20) headspace at 37°C. Electron donors and acceptors were added to the medium from aqueous, anaerobic, sterile stock solutions. *D. hafniense* was grown fermentatively with 40 mM pyruvate, or under halorespiring conditions in the presence of 20 mM pyruvate and 20 mM 3-chloro-4-hydroxyphenyl acetate (CHPA). *Escherichia coli* DH5α was used as a host for cloning vectors. Two overproduction strains were used in this study: *E. coli* BL21(DE3), to obtain recombinant protein for purification, and *E. coli* JM109(DE3), for *in vivo* promoter activity measurements. All *E. coli* strains were grown in Luria Bertani (LB) medium at 37°C (unless stated otherwise) and kanamycin (30 μg/ml) or erythromycin (200 μg/ml) was added when appropriate.

Table 2.1 Plasmids used in this study

Plasmids	Description	Reference
pET24d	5.3-kb expression vector, pMB1 ori, Kan^R, T7 promoter	Novagen
pAK80	11.0-kb shuttle vector, p15A/*L. lactis* ori, Ery^R, promoterless l*acLM* genes	(Israelsen *et al.*, 1995)
pWUR89	*cprK1* gene cloned in frame with 6×His translational fusion in pET24d using primers BG1379/BG1380	this work
pWUR166	266-bp length *cprE-cprB* intergenic region cloned into pAK80 using BG1704 and BG1743 resulting in DB3::*lacLM* promoter fusion	this work
pWUR168	322-bp length *cprK1-cprZ* intergenic region cloned into pAK80 using BG1702 and BG1742 resulting in DB2::*lacLM* promoter fusion	this work
pWUR171	164-bp length *cprK1-cprT* intergenic region cloned into pAK80 using BG1699 and BG1782 resulting in DB1::*lacLM* promoter fusion	this work
pWUR172	$CprK1^{V192E}$ encoding plasmid, pWUR89 derivative	this work
pWUR175	pWUR166 with TTAAT(N_4)ATTAA → TTGAT(N_4)ATCAA substitution resulting in $DB3^{FNR}$::*lacLM* promoter fusion	this work
pWUR176	*cprK1* gene cloned in pET24d using primers BG1379/BG1814	this work
pWUR210	$CprK1^{C11S}$ encoding plasmid, pWUR176 derivative	this work
pWUR211	$CprK1^{C200S}$ encoding plasmid, pWUR176 derivative	this work

Table 2.2 Oligonucleotide primers used in this study

Primers	Sequence[a]	Orientation[b]
BG1379	GCGCGCCATGGCTGTTGAAGGTTTGGGCAAGG	S
BG1380	CGCGCGGATCCGAGTAATACGATGTTTGTTCAG	A
BG1699	GCGGATCCACCCATGAGAAATTTGTGCATGG	S
BG1702	GCGGATCCAGCTGCTCATTGTTCAAAGC	A
BG1704	GCGGATCCGCTATAAAAACTAAAAATGTACC	A
BG1742	GCGCAAGCTTGATAAGAAAAAGAATAAAATCATCG	S
BG1743	GCGCAAGCTTGGCCAAGGTCGCCATGACG	S
BG1748	GGTTGAGAAATTCAGGTAAAG	S
BG1749	GGATCACATACGCAAGTATTAATG	A
BG1782	GCGCAAGCTTCGTTTCACTTTGTGATATTGAC	A
BG1814	CGCGCGGATCCTAGTAATACGATGTTTGTTCAG	A
BG1942	GCAGTCTTTATGCTCCGAAATG	S
BG1943	GTGATATTGACTATACCG	A
BG1944	CCTGCTTCAAAAAATATCTCC	S
BG1945	CTAATACATAAAAAGAAGCTG	A

[a] Introduced endonuclease restriction sites are underlined.
[b] S, sense primer; A, antisense primer.

DNA isolation, cloning and site directed mutagenesis

Chromosomal DNA of *D. hafniense* was isolated by first mechanically disrupting the cells by bead-beating followed by purification using the Bio101 DNA isolation kit according to standard protocols provided by the manufacturer (Geneclean). Desired DNA fragments were PCR amplified from *D. hafniense* genomic DNA as template, digested with restriction endonucleases, purified from agarose gel and finally ligated into linearized pET24d expression vector (*cprK1* gene and its mutant derivatives) or into pAK80 (promoter fragments). A list of plasmids and primers used in this study is given in Tables 2.1 and Table 2.2, respectively. Different protocols were used to introduce single nucleotide changes in the DNA sequence: the ExSite^{TM} (Stratagene) method to create CprK1^{V192E} by replacing a GTC codon with GAG; QuickChange^{TM} (Stratagene) to replace TGT codons with TCT resulting in CprK1^{C11S} and CprK1^{C200S} mutants; and finally, the overlap extension protocol (Ho *et al.*, 1989) was used to change both half sites of the inverted repeat in DB3 from 5'-TTAAT-3' into 5'-TTGAT-3'.

Overproduction and purification of wild-type and mutant CprK1

E. coli BL21(DE3) carrying one of the pET24d derivatives (Table 2.1) was grown in LB medium at 37°C until the A_{600} reached 0.6. At this point, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium and incubation was continued at 20°C overnight (O/N) to facilitate overproduction of CprK1 in the soluble protein fraction. Cells were collected by centrifugation at 4°C, then resuspended in 1/20 volume buffer A (50 mM sodium phosphate buffer [pH 7.0], 100 mM NaCl) and disrupted by French press at 100 MPa. Cell debris was removed by centrifugation at 4°C, 16,000 × g for 30 min. The supernatant was applied to a HiPrep 16/10 Heparin FF column (Amersham Biosciences) pre-equilibrated with buffer A.

Unbound proteins were washed with 8 column volumes of buffer A, and CprK1 was subsequently step-eluted using buffer B (50 mM sodium phosphate buffer [pH 7.0], 300 mM NaCl). Fractions that contained the recombinant protein were pooled and concentrated using Vivaspin 10K molecular-weight-cutoff devices (Vivascience). Concentrated samples were applied to a Tricorn Superdex 200 10/300 GL gel filtration column (Amersham Biosciences) pre-equilibrated with buffer C (50 mM Tris-HCl [7.0], 100 mM NaCl). Denaturing gel electrophoresis of the protein samples was done in 10% polyacrylamide gel. Protein concentrations were determined using the method developed by Bradford (1976).

Electrophoretic mobility shift assay (EMSA)

Three *D. hafniense* promoter regions were PCR amplified using *Pfu* DNA polymerase from *D. hafniense* genomic DNA as template. The 60-bp *cprK1-cprT* intergenic region with dehalobox DB1 was amplified by primers BG1942 and BG1943. The 63-bp *cprK1-cprZ* intergenic region with dehalobox DB2 was amplified using primers BG1944 and BG1945. Dehalobox DB3 centred in a 52-bp *cprE-cprB* intergenic region was amplified using primers BG1748 and BG1749. PCR-amplified fragments were loaded on a 10% non-denaturing polyacrylamide gel, excised and extracted by a modified "crush and soak" method. NEW buffer (10 mM Tris-HCl [pH 8.0], 750 mM NaCl, 10 mM EDTA, 0.5% SDS, 40 µg/ml glycogen) was used to incubate the crushed gel piece O/N at 50°C while continuously shaking (1400 RPM). After incubation, the gel pieces were separated from the aqueous phase by using a Bio-Spin chromatography column (Bio-Rad) then the supernatant was treated with phenol-chloroform followed by ethanol precipitation of DNA. The promoter fragments were radioactively labelled at the 5' end using [γ-^{32}P]ATP and T4 polynucleotide kinase. Unincorporated radioactive nucleotides were removed with the help of Bio-Spin P30 columns (Bio-Rad). Purified CprK1 protein for EMSA experiments was obtained from fractions eluted from the heparin chromatography column and buffer exchanged into 50 mM Tris-HCl buffer (pH 7.5). EMSA reaction mixtures (20 µl) contained 1 x POP buffer (20% glycerol, 50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 2.5 mM EDTA, 250 mM NaCl), 1 mM DTT, 1 µg poly(dG-dC)-poly(dG-dC), 25 µM CHPA or 250 µM HPA, purified CprK1 and approximately 1 nM ^{32}P-labelled DNA. The reaction mixtures were first incubated at 24°C for 30 min to allow complex formation, then loaded on a 6% polyacrylamide gel buffered with 89 mM Tris and 89 mM boric acid (app. pH 8.1) and electrophoresed at a constant current of 10 mA at 4°C, followed by drying and autoradiography.

In vivo promoter probe assay

E. coli JM109(DE3) cells were co-transformed with a pET24d expression vector carrying wild-type *cprK1* gene or one of its mutated derivatives and a pAK80 plasmid carrying either one of the DB1::*lacLM*, DB2::*lacLM*, DB3::*lacLM* or DB3^{FNR}::*lacLM* promoter fusions (Table 2.1). Each experiment was carried out in triplicates.

50 ml LB medium was supplemented with kanamycin (30 µg/ml) and erythromycin (200 µg/ml) and inoculated with 1% O/N-grown bacterial culture, then placed on a rotary shaker at 37°C until an A_{600} of 0.3 was reached. At this point, 0.1 mM IPTG was added to the culture, as well as 20 mM CHPA when appropriate, and a time zero sample was taken. The Erlenmeyer flasks were transferred to a 20°C incubator, and were continuously shaken O/N. β-Galactosidase activity was measured as described by Sambrook *et al.* (1989), except that prior to measurement of adsorption of *o*-nitrophenol at 420 nm, reaction mixtures were centrifuged for 2 minutes at 14,000 × g to remove cell particles. Hence, correction with the A_{550} values was not necessary. Throughout this study, 1 Miller unit was defined as follows: $1000 \times A_{420} / (t \,.\, v \,.\, OD_{600})$ where t is reaction time, v is sample volume and OD_{600} is the optical density of the culture at the time the sample was taken.

Results and Discussion

Desulfitobacterium hafniense cpr gene cluster

The gene coding for CprK1 (protein accession number ZP_00558871.1) is located in the 7-kb chlorophenol reductive dehalogenase gene cluster of the *Desulfitobacterium hafniense* genome (Fig. 2.1a). In analogy to the situation in *D. dehalogenans* (Smidt *et al.*, 2000), members of the *cpr* gene cluster code for proteins with known or putative function in halorespiration: *cprT* encodes a putative trigger factor (proline *cis/trans* isomerase), the product of *cprE* has homology to GroEL-type chaperons, CprB is a probable membrane anchor for the product of the downstream *cprA1*, a CHPA reductive dehalogenase (Christiansen *et al.*, 1998) and *cprC* is expected to code for a NosR/NirI-type transcriptional regulator. Apart from the orthologous gene in the *D. dehalogenans* *cpr* gene cluster, no homologue of *cprZ* could be identified in sequence databases.

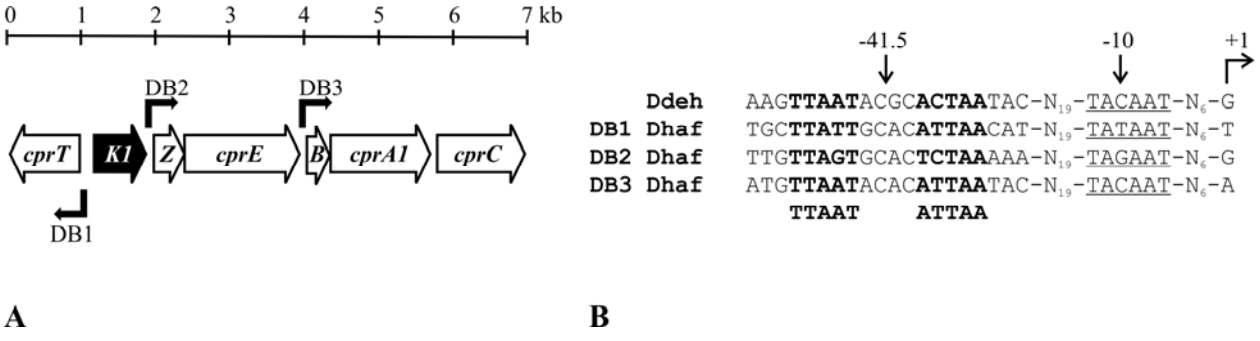

Figure 2.1 (**A**) *Desulfitobacterium hafniense* chlorophenol reductive dehalogenase gene cluster. (**B**) Alignment of three *D. hafniense* intergenic regions containing 14-bp inverted repeats termed "dehaloboxes" (DB1 to DB3) with the corresponding *cprE-cprB* intergenic region from *D. dehalogenans*. Position of dehaloboxes (boldfaced), putative Pribnow-boxes (underlined) and transcription start site previously mapped in *D. dehalogenans* are indicated with arrows above the DNA sequence alignment. Proposed consensus sequence for dehaloboxes is included below the sequence alignment.

The deduced amino acid sequence of the protein encoded by *cprK1* is 232 aa in length and has 26.5 kDa molecular weight with an isoelectric point of 8.1. CprK1 shows 94% sequence similarity (89% identity) to CprK from *D. dehalogenans*, 41% similarity (20% identity) to FNR and 44% similarity (21% identity) to CRP from *Escherichia coli.* Three putative CprK1 binding sites (*d*ehalo*b*oxes) were identified as 14-bp inverted repeats in the intergenic regions of *cprT-cprK1* (DB1), *cprK1-cprZ* (DB2) and *cprE-cprB* (DB3) (Fig. 2.1b). They all contain a 5-bp palindromic sequence that is separated by 4 spacing nucleotides. The deduced consensus sequence for dehaloboxes is TTAAT ----ATTAA, which closely resembles the FNR-consensus TTGAT----ATCAA (Guest *et al.*, 1996) and more distantly resembles the target sequence TGTGA------TCACA of CRP (Kolb *et al.*, 1993). The three dehaloboxes are centred -41.5 relative to putative Pribnow-boxes (-10), suggesting that these promoters share similar characteristics with class II promoters (Busby *et al.*, 1997). Sequence alignment of *D. hafniense* dehaloboxes (DB1, DB2 and DB3) with the corresponding *cprE-cprB* intergenic region from *D. dehalogenans,* for which the transcription start site has been mapped (Smidt *et al.*, 2000)(Fig. 2.1b), further confirms that these promoters belong to class II.

Overproduction and purification of CprK1

A 699-bp DNA fragment coding for CprK1 was PCR-amplified from *D. hafniense* genomic DNA using primers BG1379 and BG1814 and cloned into a pET24d overexpression vector resulting in plasmid pWUR176. *E. coli* BL21(DE3) cells carrying pWUR176 were grown aerobically until early-exponential-growth phase; then overproduction of CprK1 was induced upon addition of 0.1 mM IPTG, followed by O/N cultivation at 20°C. The recombinant protein contributed to approximately 15% of the total cell protein content, and O/N cultivation of the cells at low temperature resulted in approximately 50% CprK1 to be produced in the soluble cytoplasmic fraction.

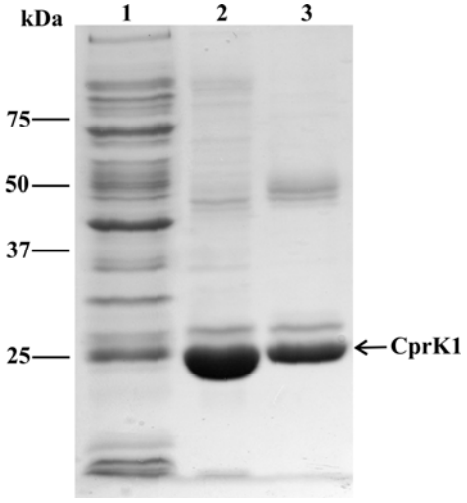

Figure 2.2 Purification of CprK1. SDS-PAGE gel showing consecutive steps of CprK1 purification: Lane 1, cell free extract (35 µg); lane 2, heparin affinity chromatography (35 µg); lane 3, gel filtration chromatography (12 µg).

Purification of CprK1 was done in two steps (Fig. 2.2). First, cell free extract was applied on a HiPrep heparin column, a general affinity chromatography matrix for DNA-binding proteins. Weakly bound CprK1 was eluted by increasing the concentration of NaCl from 100 mM to 300 mM. Pooled fractions that contained CprK1 were concentrated and applied to a Tricorn Superdex gel filtration column. The total yield of purified CprK1 was 3 mg protein per liter bacterial culture. Purified recombinant CprK1 had the expected molecular weight of 26.5 kDa (estimated from SDS-PAGE gel). Gel filtration experiments indicated that under these conditions CprK1 exists as a dimer (data not shown).

In vitro DNA-binding properties of CprK1

The interaction between the putative transcriptional regulator CprK1 and DNA was first investigated in detail by electrophoretic mobility shift assay (EMSA) using a 52-bp *cprE-cprB* intergenic region that contained dehalobox DB3, a 14-bp perfect inverted repeat (TTAATacacATTAA), as putative binding target. Binding reactions with increasing amounts of purified CprK1 protein were carried out in the presence of 25 μM CHPA, a compound that is known to be a substrate for the CHPA reductive dehalogenase enzyme encoded by the downstream located *cprA1* gene. Formation of protein-DNA complexes was observed at as low as 10 nM CprK1 concentration, indicating a high affinity of CprK1 towards its target DNA (Fig. 2.3). All target DNA was in the complexed form when 200 nM CprK1 was added to the EMSA binding mixture. By calculating the ratio between free DNA and CprK1-bound DNA, a dissociation constant (K_D) of approximately 90 nM was determined for the CprK1-DB3 complex. Binding of CprK1 to DNA was only observed in the presence of CHPA. When CHPA was omitted from the binding reaction or when it was replaced by its dechlorinated derivative, 4-hydroxyphenylacetic acid (HPA), we could not observe any protein-DNA complex formation, which indicates a very specific allosteric effect of CHPA on CprK1. Titrating a constant amount of CprK1 (150 nM) with an increasing amount of CHPA in the binding mixtures (from 0.25 μM to 50 μM) resulted in a binding constant of CHPA to CprK1 of approximately 0.4 μM. These results correlate well with that of obtained for *D. dehalogenans* CprK (Pop *et al.*, 2004). CprK shows high affinity (K_D, 190 nM) to a 193-bp *cprB* promoter fragment from *D. dehalogenans* that contains a TTAATacgcACTAA inverted repeat. DNA-binding of CprK was only observed in the presence of CHPA and not with HPA or 2-Cl-PA or cAMP. The affinity of CprK to CHPA was determined under two conditions: in the presence of target DNA (K_D, 0.4 μM) and by isothermal titration calorimetry measurement in the absence of DNA (K_D, 3.4 μM) (Pop *et al.*, 2004).

Besides the detailed study of the interaction between DB3 dehalobox and CprK1, we also carried out EMSA experiments with two other predicted target sites for CprK1. These target sites are: dehalobox DB1 in the centre of the 60-bp intergenic region of *cprT-cprK1*, and dehalobox DB2 in the centre of the 63-bp intergenic region of *cprK1-cprZ*. The binding reactions were carried out in the presence of 25 μM CHPA, 50-90 cps ^{32}P-labelled DNA and 100 nM CprK1.

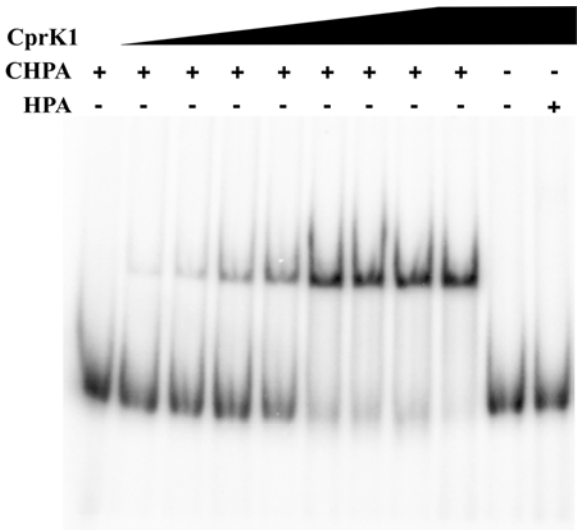

Figure 2.3 Interaction of purified CprK1 with the *cprE-cprB* intergenic region containing dehalobox DB3. Electrophoretic mobility shift assay was performed in the presence (+) or absence (-) of 25 μM CHPA or 250 μM HPA using approximately 1 nM ^{32}P-labelled DNA and increasing amounts of CprK1 protein. Concentration of CprK1 in lanes are as follows, from left to right: 0, 10, 20, 50, 100, 200, 300, 500, 1500, 1500, 1500 nM.

The results clearly show that all intergenic regions contain a binding site for CprK1, but under the conditions used CprK1 shows different affinity towards them (Fig. 2. 4). CprK1 binds DB1 dehalobox with similar affinity as it does the well-studied DB3, while clearly less protein-DNA complex is formed with DB2 as target DNA sequence at the same CprK1 concentration. The optimal binding site (DB3) for CprK1 contains a perfect inverted repeat TTAAT----ATTAA that we propose to be the consensus sequence for dehaloboxes. Deviation from this consensus is likely to influence the DNA-binding affinity of CprK1. DB1 has one mismatch at position 4 compared to the consensus sequence (T replaces the preferred A), while DB2 as the least optimal binding site has two substitutions (A is replaced by G and T replaced by C at the twofold-symmetry-related positions 4 and 11, respectively) (Fig. 2.1). Decreased binding affinity is probably caused by the elimination of a positive interaction (H bond) between the side chain amino group of Arg196 and the C-2 atom of thymine at position 4 and 11 when G·C/C·G base pairs replace the A·T/T·A base pairs. The identity of the four bases between the inverted repeats can also influence the binding affinity although they do not directly interact with the regulator protein (Scott *et al.*, 2003). However, findings that FNR-regulated class I or class II promoters have highest activity when the middle two base pairs in the spacing nucleotides are A or T are not applicable in the case of the dehaloboxes studied here.

In vivo interaction of CprK1 with E. coli RNA polymerase

We have shown that CprK1 can bind to its target dehaloboxes (DB1, DB2, and DB3) *in vitro* in the presence of CHPA. In order to investigate whether this protein is capable to initiate transcription in concert with the RNA polymerase holoenzyme, we have constructed a fusion

between three *D. hafniense* promoters containing DB1, DB2 or DB3 and the β-galactosidase-encoding *lacLM* genes on plasmid pAK80 (Israelsen *et al.*, 1995). The resulting promoter-fusion products were DB1::*lacLM* on pWUR171 (containing a 164-bp length *cprK1-cprT* intergenic region), DB2::*lacLM* on pWUR168 (containing a 322-bp length *cprK1-cprZ* intergenic region) and DB3::*lacLM* on pWUR166 (containing a 266-bp length *cprE-cprB* intergenic region). *E. coli* JM109(DE3) cells were co-transformed with either of the pAK80-constructs and with pWUR176 that contained *cprK1* gene under the control of an IPTG-inducible T7 promoter. During the promoter probe assay, CprK1 production in aerobically grown *E. coli* JM109(DE3) cells was induced by the addition of 0.1 mM IPTG followed by O/N growth at 20°C under two different conditions: in the presence of 20 mM CHPA or without additional effector.

The pattern of β-galactosidase expression mediated by CprK1 was similar for all the three promoters (Fig. 2.5). There was little detectable β-galactosidase activity in cells grown in the absence of effector molecules. Maximal activity was observed in cells cultured in medium that was supplemented with 20 mM CHPA. Control experiments were done with IPTG-induced cells carrying empty pET24d expression vector instead of pWUR176. Under these growth conditions, *E. coli* JM109(DE3) strain exhibits low level (4-6 Miller Units) native β-galactosidase background activity (data not shown), possibly originating from a *phospho*-β-galactosidase encoded by the *bgi* operon. The measured β-galactosidase activities correlate well with the affinity of CprK1 towards these dehaloboxes deduced by the ratio of free vs. complexed DNA in electrophoretic mobility shift assays, with DB3::*lacLM* having the highest and DB2::*lacLM* showing the lowest promoter activity.

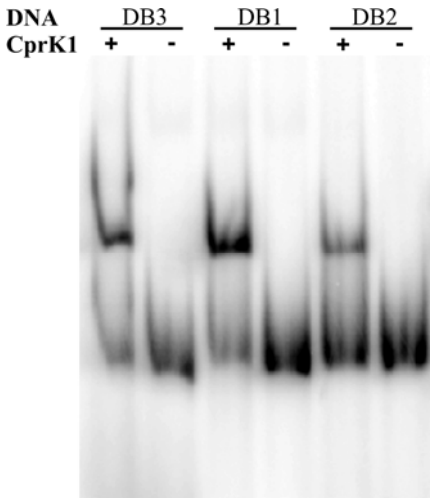

Figure 2.4 Interaction of CprK1 with dehaloboxes DB1, DB2 and DB3. Electrophoretic mobility shift assay was carried out in the presence (+) or absence (-) of 100 nM CprK1 protein, 25 μM CHPA and one of the three ^{32}P-labelled DNA containing dehaloboxes DB1, DB2 or DB3 as indicated above the gel.

In vivo promoter probe experiments showed that CprK1 is capable of CHPA-dependent initiation of transcription by enabling the recruitment of the *E. coli* RNA polymerase. The -41.5 position of the target sites (dehaloboxes) of CprK1 relative to the putative Pribnow-boxes (-10) places these halorespiration-inducible promoters within class II promoters (Fig. 2.1b). The class II-type contact between *E. coli* CRP or FNR and the RNA polymerase complex (RNAP) has been thoroughly investigated and three contact sites (activating regions [AR]) were identified in the regulators (Green *et al.*, 2001). AR1 makes contact with a conserved region ("287 determinant") of the C-terminal domain of the RNAP α-subunit (αCTD) (Benoff *et al.*, 2002), while AR2 interacts with four negatively charged amino acids of the N-terminal domain of the α-subunit (αNTD) (Busby *et al.*, 1999). The third possible interaction site (AR3) in CRP or FNR targets a conserved region in the σ^{70}-factor of the RNA polymerase holoenzyme (Lamberg *et al.*, 2002; Rhodius *et al.*, 2000). This conserved region of σ^{70} ("region 4") contains five positively charged amino acids. Sequence alignments of the corresponding subunits of the *E. coli* and *D. hafniense* RNAP show that amino acids for AR1 and AR3 contact are conserved in *D. hafniense* RNAP, while the characteristic negatively charged amino acids for AR2 contact are missing. Using sequence alignment of *D. dehalogenans* CprK and five *D. hafniense* CprK-homologues with *E. coli* CRP and FNR and the secondary structure of CRP (Schultz *et al.*, 1991), we have identified conserved amino acids that are likely to be involved in the regulatory protein-RNAP interaction (Fig. 2.6). These amino acids in CprK1 are residues 56-60 (β_3-β_4 turn in CRP) and residues 99-104 (β_8-β_9 turn in CRP) that aligned well with AR1 structures of FNR, the positively charged surface exposed residue Arg35 (AR2) and the conserved residues Ile66, Glu68, Asp69, Gly70 and Glu72, three of which are negatively charged (AR3).

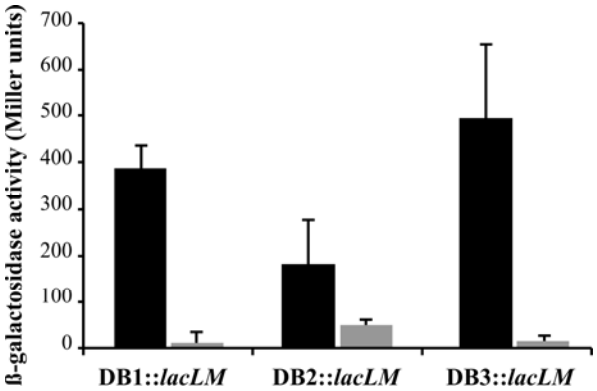

Figure 2.5 *In vivo* DNA-binding properties of CprK1. In *E. coli* cells CprK1 was overproduced in the presence of one of the three *D. hafniense* promoters containing dehalobox DB1, DB2 or DB3 fused with the *lacLM* genes. Aerobic *E. coli* cultures were grown under two different conditions: in the presence of 20 mM CHPA (solid bars) or in the absence of the effector (shaded bars). Promoter activity was expressed by measuring β-galactosidase activity (Miller units).

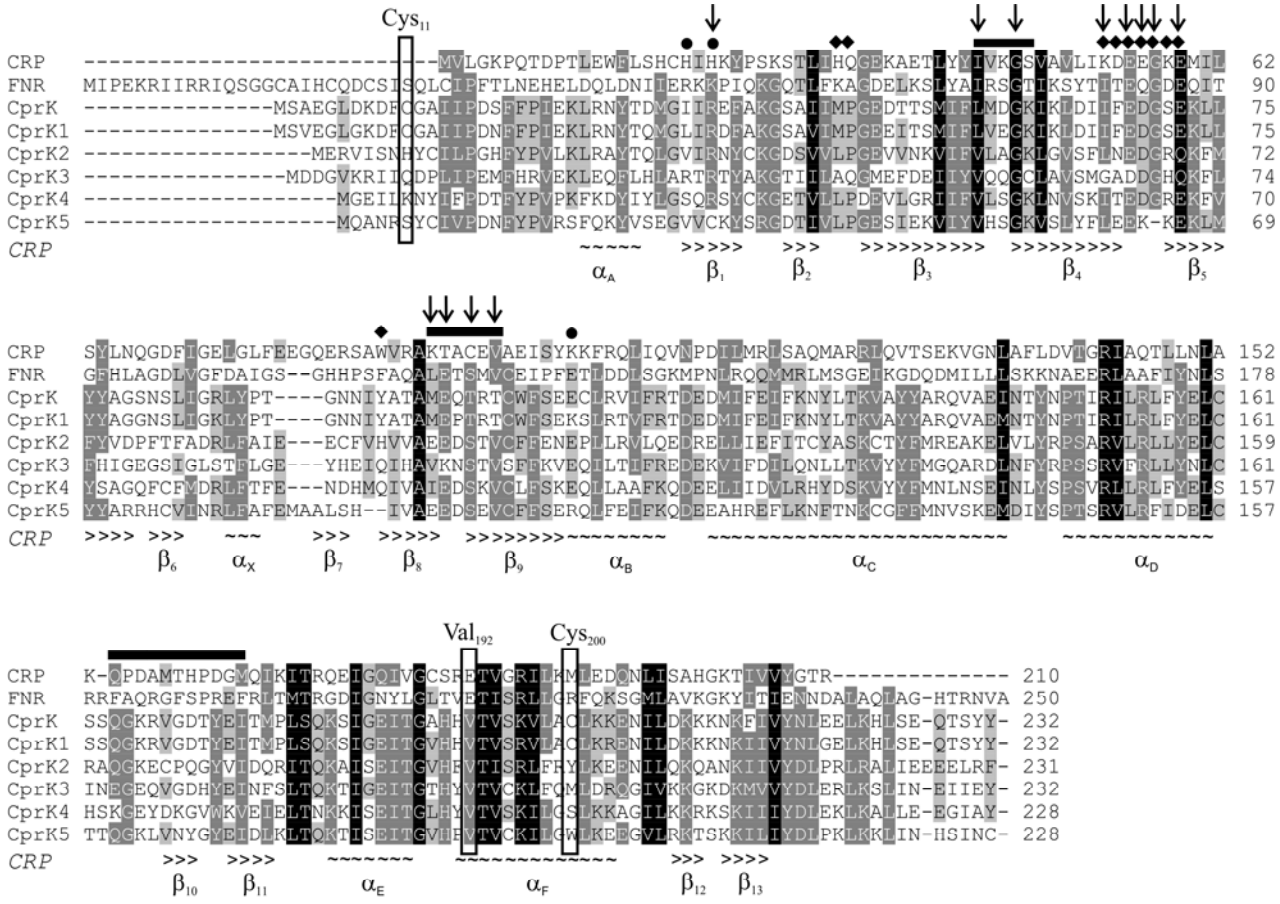

Figure 2.6 Sequence alignment of *E. coli* CRP and FNR proteins with *D. dehalogenans* CprK and five *D. hafniense* CprK homologues (CprK1 to CprK5). Essential FNR/CRP residues (activating regions, AR) previously identified to be involved in RNA polymerase contact are indicated above the alignment as follows: rectangles, AR1; circles, AR2; diamonds, AR3. The secondary structure of *E. coli* CRP is included below the sequence alignment (~, α-helix; >, β-sheet). Vertical arrows indicate amino acids that are conserved in most CprK-homologues and correspond to AR1, AR2 or AR3. In boxes, amino acids are highlighted that correspond to the position of the nonconserved Cys11 and Cys200 residues in *D. hafniense* CprK1 capable of intermolecular disulfide bridge formation, and to the position of Val192 in the recognition α-helix of CprK1 that is conserved in all CprK-homologues and corresponds to Glu181 and Glu209 residues essential for specific DNA-binding in CRP or FNR, respectively. Protein accession numbers (based on *D. hafniense* genome sequence, version of 24 June 2005) are as follows: ZP_00558871.1 (CprK1), ZP_00558892.1 (CprK2), ZP_00558887.1 (CprK3), ZP_00560926.1 (CprK4), and ZP_00559167.1 (CprK5).

Transcriptional activation by an FNR-type mutant CprK1^{V192E} protein

The dehalobox consensus sequence (TTAAT----ATTAA) is highly similar to the consensus binding sequence of FNR (TTGAT----ATCAA) with the exception that at the twofold-related positions 3 and 12, the base pairs A·T / T·A are conserved in all dehaloboxes (Fig. 2.1b), while base pairs G·C / C·G are present without exception in FNR-boxes. Recognition by wild-type FNR is abolished if TTGAT----ATCAA motif is changed into TTAAT----ATTAA by a base pair substitution at positions 3 and 12 (Bell *et al.*, 1990). It is known that in the recognition α-helix of FNR, Glu209 is involved in making essential hydrogen bonds with cytosine at positions 3 and 12 of the FNR-box sequence (Green *et al.*, 2001). This glutamate (E^{209}--RS) is highly conserved in other FNR-homologues. However, at the same position in CprK1 and all other CprK-homologues a valine is present (V^{192}--RS) (Fig. 2.6). In order to see if a single amino acid change in the recognition α-helix of CprK1 would result in altered DNA target sequence specificity, we have substituted valine at position 192 by a glutamate. CprK1 was mutated by replacing a GTC codon with GAG in pWUR89, a plasmid carrying wild-type *cprK1* gene cloned in frame with a 6×His translational fusion. The resulting pWUR172 plasmid codes for CprK1^{V192E}, a protein that has FNR-type conserved residues in its recognition α-helix (E^{192}--RS).

E. coli JM109(DE3) cells were co-transformed with pWUR166 containing DB3::*lacLM* promoter fusion and either pWUR89 or pWUR172 plasmids. Cells were grown aerobically until mid-exponential growth phase when production of wild-type CprK1 or CprK1^{V192E} was induced by addition of 0.1 mM IPTG, and cells were allowed to grow O/N at 20°C in the presence of 20 mM CHPA. Activation of transcription by CprK1^{V192E} from the TTAATacacATTAA motif containing promoter was completely abolished (Fig. 2.7). The measured β-galactosidase activity in cultures overproducing CprK1^{V192E} was not higher than the control background level.

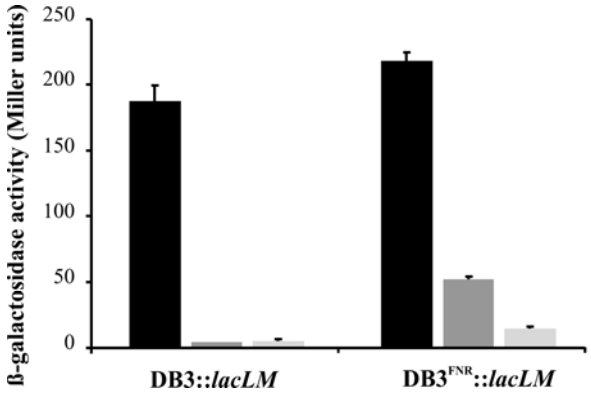

Figure 2.7 Transcriptional activation by the FNR-type mutant CprK1^{V192E}. *In vivo* promoter probe assays were carried out with aerobically grown *E. coli* JM109(DE3) cells overproducing wild-type CprK1 (solid bars); or CprK1^{V192E} (dark shaded bars) or carrying empty pET24d expression vector (light shaded bars) . All experiments were done in the presence of 20 mM CHPA with cells carrying either DB3::*lacLM* promoter fusion or DB3^{FNR}::*lacLM* promoter fusion.

In order to see if the Val192Glu substitution in CprK1 has enabled this protein to recognise an FNR-box, we mutated DB3 dehalobox (TTAATacacATTAA) at both half-sites of the inverted repeat into a consensus FNR-binding site resulting in $DB3^{FNR}$ (TTGATacacATCAA). A 266-bp length *cprE-cprB* intergenic region containing $DB3^{FNR}$ was fused to the promoterless *lacLM* reporter genes. The resulting construct, designated pWUR175, is identical to pWUR166 except for the single base pair substitutions in each half-side of the dehalobox. The loss of activity of $CprK1^{V192E}$ was restored more than 13-fold (to approximately 25% activity of the wild-type CprK1) on a promoter containing TTGAT----ATCAA motif in $DB3^{FNR}$::*lacLM* (Fig. 2.7). Interestingly, wild-type CprK1 showed similar activity on promoters containing either DB3 or $DB3^{FNR}$, suggesting the lack of preference of Val192 towards A·T or G·C base pairs at position 3 and T·A or C·G base pairs at position 12 in its target sequence. Likewise, an FNR mutant in which Glu209 was replaced by a valine gained also the ability to interact with an altered FNR-box containing A·T at position 3 and T·A at position 12 (Bell *et al.*, 1994).

Similar observations were made on a mutant CRP protein. In the recognition α-helix of CRP a glutamate at position 181 (RE^{181}---R) is essential for the recognition of the G·C base pair at position 4 and C·G at position 13 in its target sequence TGTGA------TCACA (Green *et al.*, 2001) (Fig. 2.6). The twofold-related positions 4 and 13 in CRP-boxes correspond to position 3 and 12 in FNR- and dehaloboxes. It was shown that replacement of Glu181 in CRP by valine or leucine results in the complete elimination of specificity between G·C, A·T, C·G and T·A bases at the corresponding position 4 and 13 (Ebright *et al.*, 1987).

It is very likely, that CprK1 could recognise a motif with any of the four nucleotides at position 3 and 12. However, despite of the lack of a favourable interaction of Val192 in CprK1 with any of the nucleotides, a strong conservation of A·T base pair at positon 3 and T·A base pair at position 12 occurs in dehaloboxes (Fig. 2.1). Another transcriptional regulator, NtcA from *Anabaena* sp. strain PCC 7120 shares similar features. In NtcA a valine is conserved at the position that corresponds to Val192 in CprK1, and the deduced NtcA-binding DNA motif (TGTA--------TACA) has also conserved A·T / T·A base pairs in corresponding positions 4 and 13 (Jiang *et al.*, 2000).

A homology search showed that the genome sequence of *D. hafniense* contains at least two FNR-homologues (protein accession number ZP_00560130 and ZP_00558676) with E--RS motif in their recognition α-helixes that can probably recognise an FNR-box but not a dehalobox. We hypothesise that the conservation of A·T / T·A base pairs at the twofold-related positions 3 and 12, respectively, was fixed in dehaloboxes to eliminate cross-talk between structurally related but functionally different CRP/FNR-type regulators, i.e. to avoid false induction of halorespiration genes by these FNR-homologues. On the other hand, since wild-type CprK1 is theoretically able to bind both to dehaloboxes and to FNR-boxes in the presence of CHPA (Fig. 2.7), the same issue of cross-talk mediated by CprK1 remains open. Since the two FNR-like proteins in *D. hafniense* do not contain the conserved cysteine residues of *E. coli* FNR (except for a cysteine in ZP_00560130 that corresponds to Cys122 of *E. coli* FNR) (Green *et al.*, 1993), they are not likely to sense redox changes through the incorporation of an Fe-S cluster. Thus, the identity of possible effector molecules for these FNR-homologues, as well as their target genes, remains a question.

It is likely, that the target promoters of the FNR-homologues contain more than one regulator binding site (complex bacterial promoters, reviewed by Barnard *et al.*, 2004), therefore cross-talk of CprK1 with other FNR-homologues can be prevented either by the existence of a repressor that does not allow binding of, and transcriptional initiation by CprK1 on FNR-regulated promoters; or alternatively, transcriptional initiation requires the interaction of two regulators that is only possible between the FNR-homologue and the unknown regulator and not with CprK1.

Is CprK1 subject to redox regulation?

The crystal structure of *D. hafniense* CprK1 shows the presence of two intermolecular disulfide bridges that connect Cys11 of monomer A with Cys200 in monomer B and vica versa (Joyce *et al.*, 2006). Electrophoretic mobility shift assays have already indicated that oxidized CprK1 is not able to bind its target DNA (data not shown), an observation similar to those made with CprK from *D. dehalogenans* by Pop *et al.* (2004). Since these experiments were performed *in vitro*, the question remained whether the Cys11-Cys200 disulfide bridge in CprK1 is physiologically relevant. In other words, is CprK1 subject to redox regulation in the cytoplasm by a disulfide-thiol exchange reaction? If this were the case, then substitution of the two cysteine residues with serine would result in a constantly active CprK1 protein that is insensitive to oxidation and activates transcription *in vivo* more efficiently than wild-type protein.

We have created two single amino acid substituted mutants of CprK1 by replacing a TGT codon with TCT in pWUR176 resulting in pWUR210 plasmid that encodes CprK1^{C11S} variant and pWUR211 plasmid that encodes CprK1^{C200S} protein. Both mutant proteins were overproduced and purified. SDS PAGE gel electrophoresis of protein samples that were heat-denatured in the presence or absence of 5% β-mercaptoethanol proved that CprK1^{C11S} and CprK1^{C200S} are not capable of intermolecular disulfide bridge formation (Fig. 2.8a). However, gel filtration experiments showed that CprK1^{C11S} and CprK1^{C200S} still exist as a homodimer in solution, which indicated that the disulfide bridge is not essential for dimer formation. To test their *in vivo* activity, we co-transformed *E. coli* JM109(DE3) cells with pWUR166 containing DB3::*lacLM* fusion and either pWUR210 or pWUR211. *E. coli* cells were grown aerobically until mid-exponential phase, then CprK1 production was induced by addition of 0.1 mM IPTG and was continued in the presence or absence of 20 mM CHPA at 20°C. β-galactosidase activity was measured after O/N growth. There was no significant difference between the transcriptional activation mediated by wild-type CprK1 and CprK1^{C11S} (Fig. 2.8b). The mutant CprK1^{C200S} had 340% of the wild-type activity in the presence of CHPA, but its effector-free activity was also significantly higher (28×) than the wild-type background, indicating most likely that CprK1^{C200S} mutant was overproduced relatively better in the soluble cytoplasmic fraction than the wild-type or the CprK1^{C11S} mutant. This hypothesis is supported by *in vitro* observations in EMSA experiments where CprK1^{C200S} did not show increased DNA-binding affinity compared to equal amounts of wild-type CprK1 under reducing conditions (data not shown).

As conclusion, *in vivo* transcriptional activation experiments by CprK1 and its single amino acid substituted derivatives CprK1^{C11S} and CprK1^{C200S} suggest that recombinant CprK1 does not contain a disulfide bridge in the cytoplasm of the aerobically cultivated *E. coli* cells.

There is further evidence that support this hypothesis. First of all, the midpoint redox potential of *E. coli* cytoplasm is ≤ -250 mV (Unden *et al.*, 1994), which strongly disfavors the formation of stable disulfide bonds in proteins. Thus it is generally accepted that in the absence of enzymatic systems (e.g. thiol-disulfide oxidoreductases), the formation of cysteine-cysteine linkages is extremely slow in the cytoplasm (Kadokura *et al.*, 2003). However, an increasing number of transcriptional regulators have been identified that undergo a temporary thiol-disulfide exchange reaction or thiol modification during oxidative stress. Active FLP from *Lactobacillus casei* contains an intramolecular disulfide bridge (Gostick *et al.*, 1998). In CrtJ-homologues two cysteine residues are conserved (Cys249 and Cys420) that form a disulfide bridge in the active CrtJ protein characterised from *Rhodobacter capsulatus* (Masuda *et al.*, 2002). OxyR from *E. coli* is a well-studied regulator of genes involved in anti-oxidative response (Aslund *et al.*, 1999).

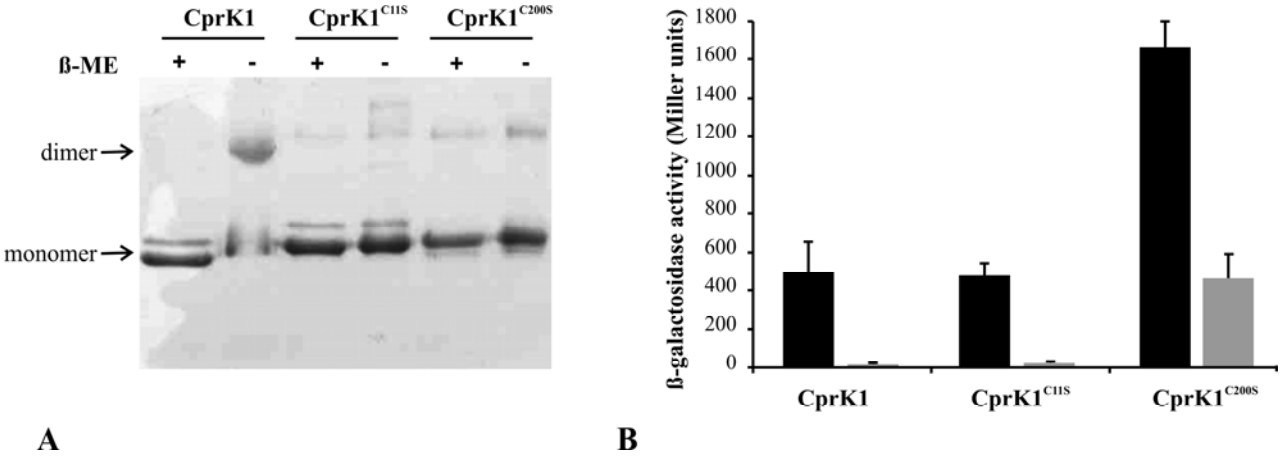

Figure 2.8 Effect of redox state of CprK1 on its activity. (**A)** SDS PAGE gel of purified wild-type CprK1, CprK1^{C11S} and CprK1^{C200S} proteins (5.8 µg) that were heat denatured in the presence (+) or absence (-) of 5% β-mercaptoethanol (β-ME). CprK1^{C11S} and CprK1^{C200S} proteins contain a C-terminal 6×His tag causing slight difference in their mobility compared to wild-type CprK1 on SDS-PAGE gel but leaving other properties of the proteins unaffected. (**B**) *In vivo* promoter probe assay using *E. coli* JM109(DE3) cells harbouring pWUR166 plasmid with DB3::*lacLM* promoter-fusion and pET24d plasmid derivative that overproduces either wild-type CprK1, CprK1^{C11S} or CprK1^{C200S} proteins. Cells were grown in presence of 20 mM CHPA (solid bars) or in the absence of the effector (shaded bars).

The crystal structure of the oxidized active form of OxyR shows the presence of a disulfide bridge between Cys199 and Cys208, two residues that are 17 Å apart in the reduced protein (Choi *et al.*, 2001). Kim *et al.* (2002) questioned the physiological relevance of this disulfide bridge in activation of the protein and regarded it merely as an artifact of the lengthy crystallization process. Instead, they proposed a multiple activation state model where Cys199 of OxyR can be modified into a sulfenic acid, S-nitrosothiol or mixed glutathione disulfide intermediate according to different environmental signals. A similar event could have occurred during the crystallization of CprK1 under aerobic conditions, resulting in the formation of the possibly non-physiological intermolecular disulfide bridges between Cys11 and Cys200. These residues are only conserved in *D. hafniense* CprK1 and in *D. dehalogenans* CprK and not in other *D. hafniense* CprK-homologues, although these proteins do show an unusually high cysteine content (Fig. 2.6). The only cysteine that is shared among four CprK-homologues (including CprK1) and CprK is Cys105 that corresponds to Cys122 of FNR from *E. coli*. This cysteine is essential for intramolecular disulfide bond formation and involved in Fe-S cluster binding in FNR (Green *et al.*, 1993). Pop *et al.* (2004) have investigated the role of Cys105 by replacing this residue with alanine in *D. dehalogenans* CprK. Transcriptional activation mediated by the C105A mutant was compared with that of the wild-type CprK, which led to the conclusion that this mutation has no effect on the activity of this protein.

Although we have shown that there is no evidence of disulfide bond formation in recombinant CprK1 when it is produced aerobically in *E. coli*, we cannot exclude the possibility that this protein gives a different response in *D. hafniense* when the cells are exposed to oxygen. It was reported that although no growth occurs in its presence, *D. hafniense* can tolerate oxygen and resume growth in anaerobic media after oxygen exposure for 24 h (Madsen *et al.*, 1992). In *D. hafniense* there are redox proteins (iron-sulphur proteins, flavins, flavoproteins and quinones) that are potentially capable of carrying out the one-electron reduction of oxygen to superoxide, and possibly further to hydrogen peroxide. One of the redox proteins is the CHPA reductive dehalogenase (CprA1) itself, encoded in the same *cpr* gene cluster as CprK1. This enzyme contains a corrinoid cofactor and three [4Fe-4S] redox centres that are normally involved in the electron transfer during the reductive dehalogenation of CHPA to HPA (Christiansen *et al.*, 1998). Accumulation of the highly reactive superoxide and hydrogen peroxide radicals would be toxic for the cells, so a likely defence mechanism can involve the down-regulation of the transcription of *cpr* genes by oxidative inactivation of CprK1 (Pop *et al.*, 2004). However, *D. hafniense* cells have the capacity to directly deal with these radicals. We have found two superoxide dismutase and two catalase-encoding genes in its genome sequence and a gene whose product shows high homology to a peroxidase/catalase from *Geobacillus stearothermophilus*. Madsen *et al.* (1992) detected catalase activity in *D. hafniense* cells, further confirming that *D. hafniense* directly inactivates reactive radicals produced upon O_2 exposure rather than preventing their formation, as that would require the down-regulation of several redox proteins.

In conclusion, our results indicate that heterologously overproduced CprK1 does not contain a Cys11-Cys200 disulfide bridge *in vivo*. Consequently, in *D. hafniense* this protein is most likely to be the subject of activation only by CHPA and not by disulfide to thiol reduction.

Acknowledgements

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CprK crystal structures reveal mechanism for transcriptional control of halorespiration

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Abstract

Halorespiration is a bacterial respiratory process in which haloorganic compounds act as terminal electron acceptors. This process is controlled at transcriptional level by CprK, a member of the ubiquitous CRP-FNR family. We here present the crystal structures of oxidized CprK in presence of the ligand *ortho*-chlorophenolacetic acid and of reduced CprK in absence of this ligand. These structures reveal that highly specific binding of chlorinated – rather than the corresponding non-chlorinated – phenolic compounds in the N-terminal β-barrels causes reorientation of these domains with respect to the central α-helix at the dimer interface. Unexpectedly, the C-terminal DNA-binding domains dimerize in the non-DNA binding state. We postulate that the ligand-induced conformational change allows formation of interdomain contacts that disrupt the DNA-domain dimer-interface and leads to repositioning of the helix-turn-helix motifs. These structures provide a structural framework for further studies on transcriptional control by CRP-FNR homologs in general and of halorespiration regulation by CprK in particular.

Introduction

Past and present industrial and agricultural activities have led to the ever increasing presence of haloorganic compounds such as chlorophenols and chlorinated ethenes in the environment (Oberg, 2002). Due to both toxic and recalcitrant nature, increasing amounts of these xenobiotics threaten integrity of the environment and human health (Henschler, 1994). In recent years, it has emerged that several organohalids are also naturally produced (Gribble, 2003), and that several species of strictly anaerobic bacteria are able to conserve energy via the reductive dehalogenation of these compounds by respiratory metabolism (Smidt & de Vos, 2004; Van de Pas *et al.*, 2001). In view of their favourable degrading capacities, e.g. high dehalogenation rate and low residual concentration of the contaminant, it has been anticipated that halorespiring microorganisms should be of utmost significance for efficient biological remediation of halogenated hydrocarbons in anoxic environments (El Fantroussi *et al.*, 1998; Furukawa, 2003). The versatile, strictly anaerobic gram-positive bacterium *Desulfitobacterium dehalogenans* and the closely related *Desulfitobacterium hafniense* have the capacity of degrading *ortho*-chlorophenol. Both have been used as model organisms in halorespiration studies, representing one of the most significant groups of halorespiring isolates (Van de Pas *et al.*, 1999). In these organisms, proteins involved in halorespiration are encoded by the *cpr* (*c*hlorophenol *r*eductive dehalogenase) operon, of which multiple copies are present within the genome. This potentially allows for reductive dehalogenation of a wide range of haloorganic compounds by the use of a series of paralogous enzymes (Villemur *et al.*, 2002).

The *cpr* operon is regulated on the level of transcription by CprK, a member of the CRP-FNR family of regulators that is ubiquitous in bacteria (Smidt *et al.*, 2000). Recent *in vivo* and *in vitro* studies reveal that CprK binds 3-chloro-4-hydroxyphenylacetate (CHPA) with micromolar affinity promoting a tight interaction with a specific DNA sequence in the promoter region of the

cpr-encoded genes, called the "dehalobox" (Gábor *et al.*, 2006; Pop *et al.*, 2004). CHPA-like compounds that lack either the chloride or the hydroxyl group fail to induce DNA-binding, even at millimolar concentrations. The *cpr* gene cluster appears to be subject to a second layer of transcriptional control - under aerobic conditions, oxidation of CprK leads to disulfide bond formation, which prevents DNA-binding (Pop *et al.*, 2004).

Regulators of the CRP-FNR family respond to a broad spectrum of intracellular and exogenous signals (Korner *et al.*, 2003). To accomplish their roles, these regulatory proteins have intrinsic sensory modules that either bind allosteric effector molecules or chemically modulate prosthetic groups, after which a signal is transmitted to a DNA binding domain. For example, the paradigmatic *Escherichia coli* cAMP receptor protein (CRP) undergoes a conformational change upon cAMP binding that triggers binding to the promoter region of target genes (Harman, 2001). To date, crystal structures of distinct CRP states provide atomic insights into cAMP-, DNA- and RNA-polymerase-binding (Benoff *et al.*, 2002; Lawson *et al.*, 2004; Passner *et al.*, 2000). However, a detailed structure for the ligand-free, non-DNA binding state of CRP is lacking. The structural data available for other CRP-FNR family members is limited to the crystal structures of CooA from *Rhodospirillum rubrum* (Lanzilotta *et al.*, 2000) and the pathogenicity factor PrfA from *Listeria* spp. (Eiting *et al.*, 2005), both ligand-free structures. Due to the absence of a family member with both ligand-bound and ligand-free states available, the allosteric mechanism by which effector binding induces a conformational transition to an "active", DNA-binding state is poorly understood, and several models have been put forward to explain how the sensory module can transmit the signal to the distant DNA-binding domain (Chen *et al.*, 2003; Harman, 2001; Passner *et al.*, 2000; Yu *et al.*, 2004).

We here report the 2.2 Å crystal structure of the oxidized *D. hafniense* CprK in the presence of CHPA, and the 2.9 Å crystal structure of the highly related *D. dehalogenans* CprK (both proteins are 232 amino acids long and 89% identical) in the ligand-free, reduced state. Comparison of both structures allows identification of the allosteric changes induced by ligand binding.

Materials and Methods

Crystallisation and structure elucidation of D. hafniense CprK

CprK was prepared as described previously (Gábor *et al.*, 2006), using the pWUR89 expression vector. Crystals of the oxidized CprK-CHPA complex were grown in 100 mM Tris-HCl pH 7.5, 1.8 M ammonium sulphate at 4°C. Heavy atom derivatisation of the crystals was carried out by soaking crystals in mother liquor containing 10 mM potassium tetrachloroplatinate (II) and 10 mM mercury (II) acetate. Data were collected on single crystals at DESY, Hamburg, beamline BW7A. All data were processed and scaled using DENZO and SCALEPACK (Otwinowski *et al.*, 1997) and crystals belonged to the I 222 space group (a= 104.4 Å, b=112.2 Å, c=119.5 Å) (Table 3.1). The position of heavy atoms sites for isomorphous derivatives was found using difference Patterson functions and verified using (cross) difference Fourier analysis.

Coordinates and real and anomalous occupancies were refined for all heavy atom positions and final MIRAS phases calculated using MLPHARE (Collaborative Computational Project, 1994). Density modification and NCS symmetry averaging were carried out using DM (Cowtan, 1994). Model building was carried out using TURBO-FRODO (Roussel *et al.*, 1991) and maximum likelihood refinement was carried out using REFMAC5 (Murshudov *et al.*, 1997). Final coordinates and structure factors have been deposited with the PDB (code 2H6B and RCSB037983, respectively).

Crystallisation and structure elucidation of D. dehalogenans CprK

CprK was expressed and purified as described previously (Pop *et al.*, 2004). CprK (7-8 mg/ml) was crystallized in a solution containing 100 mM MES pH 6.5, 115 mM $MgCl_2$, 8% PEG3350, and 10 mM DTT. Data were collected from a single flash-cooled crystal at beam line 11-1 at SSRL. All data were processed and scaled using Crystal Clear (CrystalClear, 1999) and crystals belonged the P21 space group (a=72.7 Å, b=50.0 Å, c=76.4 Å, β=105.5°) (Table 3.1). Molecular replacement using the individual domains of the *D. hafniense* CprK was carried out using Phaser (McCoy *et al.*, 2005). Model building was carried out using TURBO-FRODO (Roussel *et al.*, 1991) and COOT (Emsley *et al.*, 2004). Maximum likelihood and TLS refinement were carried out using REFMAC5 (Murshudov *et al.*, 1997; Winn *et al.*, 2001). Final coordinates and structure factors have been deposited with the PDB (code 2H6C and RCSB037984, respectively).

Trp fluorescence quenching

Fluorescence experiments were carried out using a Cary Eclipse Fluorescence Spectrophotometer with constant temperature at 25°C maintained using a Cary single cell peltier regulator. Samples were measured using a Hellma precision quartz cuvette with a 10-mm-light path. Excitation was carried out at 295 nm with a 5 nm wavepath and emission fluorescence measured at 370 nm with a 10 nm wavepath. CHPA was added to a 1 ml solution of 10 µM WT or mutant CprK in 50 mM potassium phosphate buffer pH 7.2. Quenching observed was corrected for the inner filter effect by titration of CHPA versus tryptophan. To determine the binding constant for 4-hydroxyphenylacetic acid (HPA), titrations versus CHPA were carried out in the presence of 1 to 10 mM HPA.

Native macromolecular mass spectrometry

A concentration of 4 µM CprK dimer was used for mass spectrometry studies in the presence of 10 mM dithiothreitol. For DNA binding measurements, 4 µM CprK was incubated with 400 µM CHPA and 5 µM dsDNA for 30 min at room temperature. Native macromolecular mass spectrometry measurements were performed in positive ion mode using an electrospray ionization time-of-flight instrument (LC-T; Micromass, Manchester, UK) equipped with a Z-spray nano-electrospray ionization source. In order to produce intact ions *in vacuo* from large complexes in

solution, the ions were cooled by increasing the pressure in the first vacuum stages of the mass spectrometer (Krutchinsky *et al.*, 1998; Tahallah *et al.*, 2001). Source pressure conditions and electrospray voltages were optimized for transmission of the macromolecular protein complexes (Tahallah *et al.*, 2001). The needle and sample cone voltage were 1400 V and 140 V, respectively. The pressure in the interface region was adjusted to 8 millibars. The spectra were mass calibrated by using a solution of 10 mg/ml cesium iodide in isopropanol:water 50:50. Deconvoluted spectra were generated by using the Transform tool of the MassLynx software.

Table 3.1 Crystallographic data collection and refinement parameters

	CprK *D. hafniense* (2H6B)	**Potassium tetra-chloroplatinate soak**	**Mercury acetate soak**	**CprK *D. dehalogenans* (2H6C)**
Soak time/ Concentration		10 min/10 mM	10 min/10 mM	
Space group	I 222	I 222	I 222	P 2_1 β=105.5°
Cell dimensions				
a (Å)	104.4	104.9	105.2	72.7
b (Å)	112.1	113.0	112.3	50.0
c (Å)	119.4	119.6	117.8	76.4
X-ray source	DESY BW7A	DESY BW7A	DESY BW7A	11.1 SSRL
Resolution (Å)	50-2.2 (2.25-2.2)	50-2.5 (2.55-2.5)	50-3.1 (3.2-3.1)	50-2.9 (2.97-2.90)
No. of observations				
Total	766,373	313,230	423,430	54,952
Unique	35,512	24,333	12,468	16,387
Completeness (%)	99.2 (100)	97.6 (98.6)	95.8 (99.8)	99.0 (99.8)
I/σI	16.6 (2.5)	17.08 (2.906)	8.97 (2.31)	10.6 (3.6)
R_{merge}	0.102 (0.573)	0.078 (0.427)	0.147 (0.415)	0.056 (0.257)
Model	Residues 3-250 chain A, 9-243 for chain B, residues 233-250 are part of the C-terminal His-tag, 2 CHPA, 2 sulphate ions, 230 water molecules			Residues 19-226 chain A, 19-226 for chain B
R_{cryst} / R_{free}	18.4(41.5)/22.9(53.6)			23.2(34.0)/30.7(44.0)
Average B-factor	39.9			70.1
Ramachandran plot: core/ allowed/ generously allowed	89.5/9.7/0.7			83.6/15.9/0.3
R.m.s.d. bond lengths (Å)	0.024			0.019
R.m.s.d. bond angles (°)	1.989			2.075

Results and Discussion

Crystal structure of oxidized, ligand-bound D. hafniense CprK

The crystal structure of oxidised *D. hafniense* CprK in complex with CHPA was determined to 2.2 Å using MIRAS and NCS averaging techniques (Fig. 3.1a). The asymmetric unit contains a single CprK dimer with each monomer folded in two distinct domains connected by a long α-helix. The root mean square deviation (r.m.s.d.) in Cα position between both monomers in the asymmetric unit is 0.455 Å for the DNA-binding domain and 0.256 Å for the sensory module. A search of the protein structure database reveals that CprK is most similar to CRP (PDB code 2CGP; Fig. 3.1c) (sensory module: Z score of 13.9 and r.m.s.d. of 3.0 Å for 124 Cα atoms; DNA-binding domain: Z score of 9.3 and r.m.s.d. of 1.9 Å for 68 Cα atoms).

The relative position of the sensory modules within the CprK dimer is similar to CRP, the dimer interface predominantly made by the C α-helices. However, in contrast to CRP and other crystal structures of related regulators, CprK is a domain swapped dimer, with the C-terminal DNA-binding domain of one monomer interacting with the N-terminal effector binding domain of the other. While a non-crystallographic two-fold axis relates both sensory modules, the relative position of both DNA-binding domains is drastically different, resulting in a distinctly asymmetric CprK dimer (Fig. 3.2a). Within the dimer, two intersubunit disulphide bonds are present between Cys11 from the sensory module and Cys200 located in the DNA-binding helix F. For both DNA-binding domains, contacts with β strands 4 and 5 of the sensory module are mainly made by residues of the C-terminal helix G (not present in CRP), leading to a surface area buried of respectively ~70 $Å^2$ and ~220 $Å^2$ for the individual contacts. In contrast, in the CRP-DNA complex (Fig. 3.1c), contacts between β strands 4 and 5 and the DNA-binding domain occur via residues provided by helices D and E. Thus, whereas in CRP both DNA binding helices are positioned roughly perpendicular to the central α-helical pair along the same face of the molecule, the CprK DNA-binding helices are near parallel to the C α-helices.

This conformation is not compatible with tight binding to the "dehalobox" DNA. Reorientation of both domains to a position similar to that observed in CRP-DNA complexes requires breaking the disulphide bond between Cys11 and Cys200 and reorientation of the N-terminal polypeptide stretch preceding Pro16. In CRP, residues from helices D and E are involved in interdomain contacts; surprisingly, the corresponding residues for both CprK DNA-domains are in close contact with the same residues from a symmetry related dimer (Fig. 3.2b).

The interface formed between two CprK dimers involves the close association of four DNA-binding domains: an 870 $Å^2$ surface that is mainly hydrophobic, containing 4 salt bridges in addition to several hydrogen bonds. The interface has a surface complementarity 0.68 (Lawrence *et al.*, 1993), suggesting that it is a physiologically relevant interaction in the non-DNA binding state.

CHPA binding by CHPA

Clear electron density is observed for a tightly bound CHPA molecule in the sensory modules of both monomers in a position similar to the binding site of cAMP in CRP (Fig. 3.3). CprK makes six direct hydrogen bonds to CHPA and two salt bridge contacts, one between Lys86 and the acetate group of the effector and another between the CHPA phenol hydroxyl group (assuming this is deprotonated) and the conserved residue Lys133. The phenol hydroxyl group forms hydrogen bonding contacts with Tyr76 (2.3 Å), Lys133 (2.7 Å) and the backbone nitrogen of Gly85 (3.0 Å) of one monomer. The chloride atom is positioned in van der Waals contact with residues of the central α-helix of the opposite monomer. The binding pocket for the chloride atom is made up of hydrophobic residues Tyr130 (3.7 Å), Leu131 (3.8 Å) and Val134 (3.9 Å) in addition to a buried water molecule (3.9 Å), several main chain atoms of Gly85 (3.7 Å) and the Lys133 amino terminal group (3.6 Å). The CHPA acetic acid group is hydrogen bonded to Lys86 (2.7 Å), Thr90 (2.6 Å) and Asn92 (2.8 Å), while the aromatic moiety is sandwiched between several hydrophobic residues in the β-barrel.

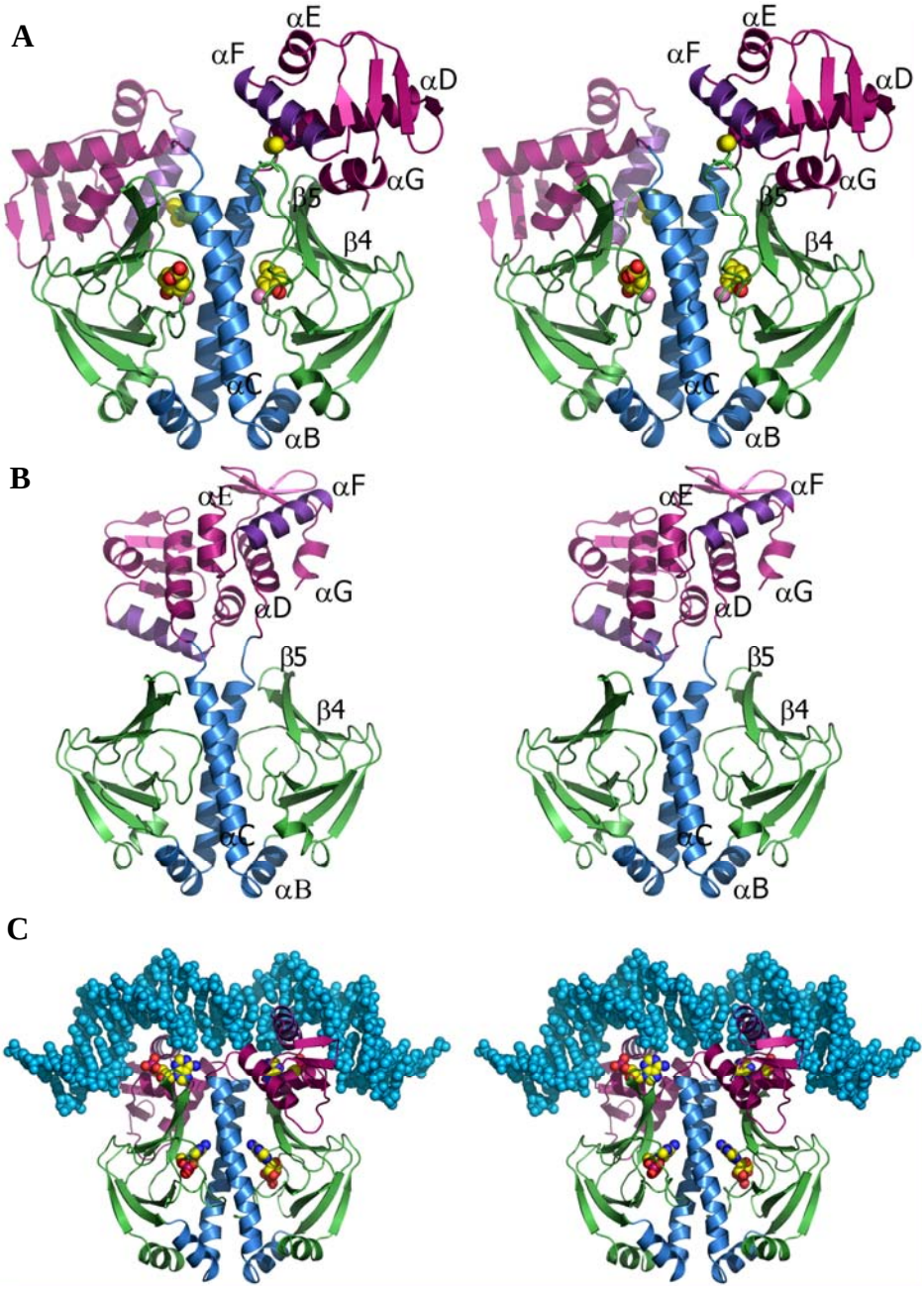

Figure 3.1 Crystal structures of CprK and comparison with cAMP-CRP-DNA complex. (**A**) Stereo view of the crystal structure of the oxidized ligand-bound CprK from *D. hafniense* in cartoon representation. The N-terminal β-barrels are coloured green, the α-helices B and C are blue, the C-terminal DNA binding domains in pink, with the exception of the DNA binding helix of the helix-turn-helix motif that is in purple. The bound CHPA and the intermolecular disulphide bond between Cys11 and Cys200 are represented in atom coloured spheres. Distinct secondary elements are labelled for domains to the right side of the dimer. (**B**) Stereo view of the crystal structure of the reduced ligand-free CprK from *D. dehalogenans* in cartoon representation. Colour coding is according to panel A. (**C**) The crystal structure of *Escherichia coli* CRP in complex with DNA and cAMP (PDB code 1CRG) in cartoon representation. Colour coding is according to panel A with representation of bound cAMP molecules in atom coloured spheres and DNA in blue spheres.

Crystal structure of reduced, ligand free D. dehalogenans CprK

The crystal structure of the reduced *D. dehalogenans* CprK was solved to 2.9 Å by molecular replacement using the individual *D. hafniense* CprK domains as search models. The asymmetric unit contains a single CprK dimer with the sensory modules and central α-helix coiled coil in highly similar orientations as observed for oxidized CprK (Fig. 3.1a,b). The r.m.s.d. in Cα position between both monomers in the asymmetric unit is 0.251 Å for the DNA-binding domain and 0.319 Å for the sensory module. Close comparison of both the ligand-free and ligand-bound sensory modules reveals no significant change in the relative position of helices B and C, which provide the majority of the CprK dimer interface (r.m.s.d. 0.3 Å for 46 Cα atoms). In contrast, the positions of both N-terminal β-barrels have shifted with respect to the B and C helices in the ligand-free structure (Fig. 3.4). The motion relating the position of the N-terminal β-barrel in both crystal structures constitutes a rigid body hinge movement around residue Ser 108, which immediately preceeds helix B. This leads to a maximum shift in position of ~3.8 Å for β strands 4 and 5. Due to the relatively small shift in position and the flexibility of some side chains, many of the direct contacts between the β-barrel and the central C helix are conserved between both conformations.

The CHPA binding site is empty (water molecules cannot be reliably observed at this resolution) and is part of a tunnel extending across the molecule and providing solvent access. While the residues involved in binding CHPA are completely conserved between both *D. hafniense* and *D. dehalogenans* CprK (except for the conservative replacement of Lys/Arg86), they occupy different conformations in both crystal structures (Fig. 3.5). A notable exception is residue Lys133, which does not show a ligation-dependent conformational shift. The 3.7 Å shift in the Cα position of Gly85 is one of the largest differences observed when comparing both structures. In contrast to the relatively minor changes observed in the sensory modules, the short linker regions connecting the C helix with the DNA-binding domain are projecting outward from the central coiled coil, positioning the DNA-binding domains at a distance from the sensory modules rather than the close contacts observed in the oxidized CprK structure. Both DNA-binding domains form a close association that buries hydrophobic residues from helices D and E and is identical to the dimerization observed between oxidized CprK dimers (Fig. 3.2b), providing further evidence for the physiological relevance of this interaction.

Native ESI-MS reveals a single CprK dimer that binds dsDNA in presence of CHPA

The ESI-MS spectrum of *D. hafniense* CprK under reducing conditions (Fig. 3.6a) revealed that CprK is mainly dimeric with a measured mass of 52,739.4 ± 3.7 Da (calculated mass 52,736 Da). Figure 3.6 shows the ESI-MS spectrum of CprK incubated with a 30-bp double stranded (ds) DNA fragment containing the "dehalobox" recognition site, and CHPA under reducing conditions. The spectrum clearly shows that dimeric CprK interacts specifically with one molecule of dsDNA (measured mass 71,165.0 ± 2.7) with very low amounts of free dsDNA and CprK present.

The observed mass increase of the analysed complex as compared to the calculated mass (71,143 Da) is likely due to water or buffer molecules still present in the protein-DNA complex. In the absence of CHPA, the level of CprK-DNA complex was very low. These results show that active CprK is a dimer that binds "dehalobox" DNA in equimolar amounts when CHPA is present.

Trp-fluorescence quenching indicate high specificity for CHPA

Trp-fluorescence quenching experiments demonstrate up to 55% quenching upon titration of oxidized *D. hafniense* CprK with CHPA, leading to an apparent K_D of 4.1 ± 0.4 μM for CHPA. This binding becomes slightly tighter (to an apparent K_D of 0.83 ± 0.11 μM) when using a Cys200Ser mutant (Fig. 3.6b, Table 3.2), which is unable to form the disulphide bond (Gábor *et al.*, 2006). A single Trp is situated at the bottom of the β-barrel, which is not in close contact with the CHPA binding site. It is therefore most likely that binding of CHPA triggers a conformational change that results in the fluorescence quenching. Titration with 4-hydroxyphenylacetic acid (HPA), which only differs from CHPA in lacking the chloride substituent, does not result in significant quenching even at millimolar concentrations. Binding of HPA to CprK can indirectly be observed through inhibition of CHPA-induced Trp-fluorescence quenching, leading to an observed K_D for HPA of 6.2 ± 0.4 mM. Thus, despite the relatively small difference in structure, CprK has a remarkable preference for the chlorinated compound, in terms of both affinity (>1000-fold higher for CHPA over HPA) and functionality (HPA-binding does not induce any measurable increase in DNA-binding). Thus, the product of CHPA respiration does not induce transcription of the *cprBA* operon that encodes a CHPA reductive dehalogenase or significantly interfere with the CHPA-induced allosteric transition.

Table 3.2 Observed K_D values for CHPA and HPA binding to *D. hafniense* CprK

Protein sample	**Ligand**	**K_D (μM)**
CprK(oxidized)	CHPA	4.1 ± 0.4
CprK(C200S)	CHPA	0.83 ± 0.11
CprK (Y76F) (oxidized)	CHPA	14.39 ± 0.79
CprK (Y76F) (reduced)	CHPA	10.24 ± 0.49
CprK	HPA	6206 ± 423

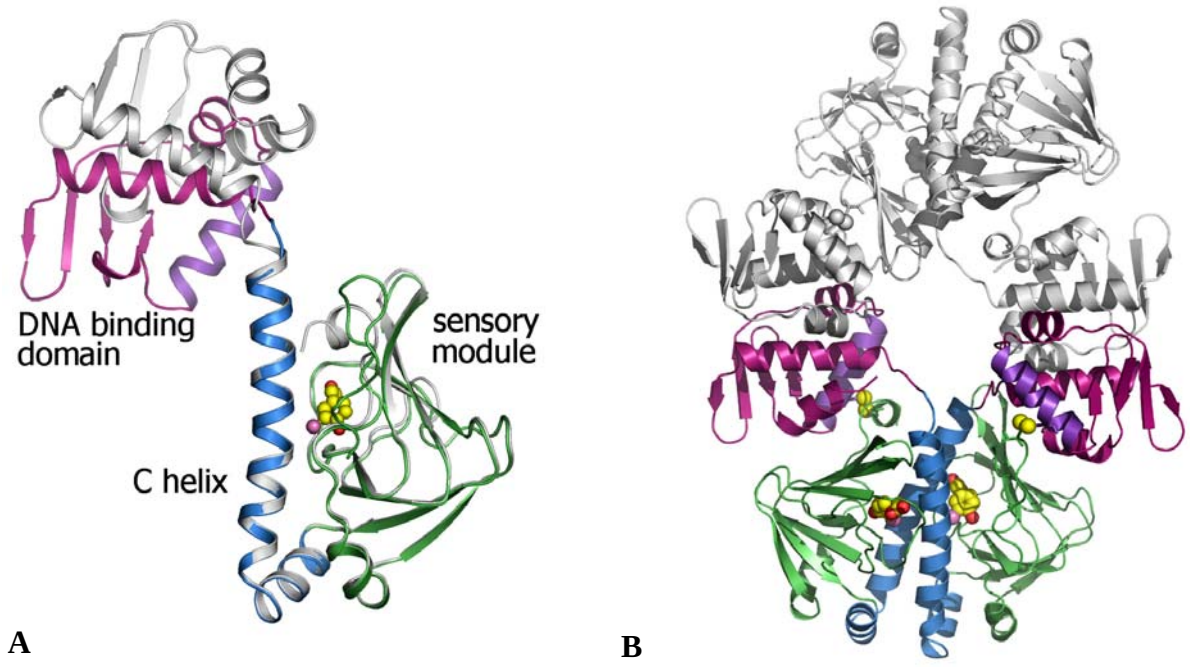

Figure 3.2 Oligomeric structure of oxidized CprK. (**A**) Overlap of both monomers within the oxidized CprK dimer observed in the asymmetric unit. One monomer is coloured grey while the other is coloured as in Fig. 3.1. (**B**) Cartoon representation of the putative tetrameric CprK molecule obtained by applying two-fold crystal symmetry, one CprK dimer is coloured grey, while the other is coloured as in Fig. 3.1.

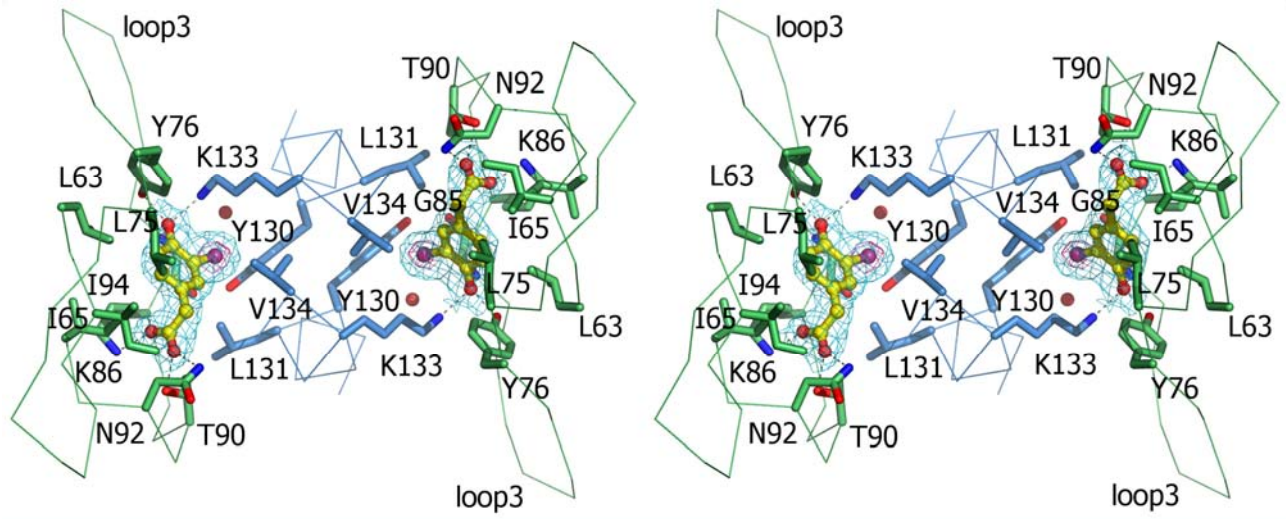

Figure 3.3 *D. hafniense* CprK CHPA binding site. Stereo representation of the binding site for CHPA with key residues depicted in sticks in addition to the Cα-trace for residues 63-94 (part of the β-barrel) and 129-135 (part of the C helix). Colour coding is according to Fig. 3.1. Hydrogen bonds between binding site residues and CHPA are indicated by black dashed lines. The SigmaA weighted F_oF_c omit map is superimposed on the bound CHPA molecules contoured at 3σ (in blue) and 6σ (in magenta).

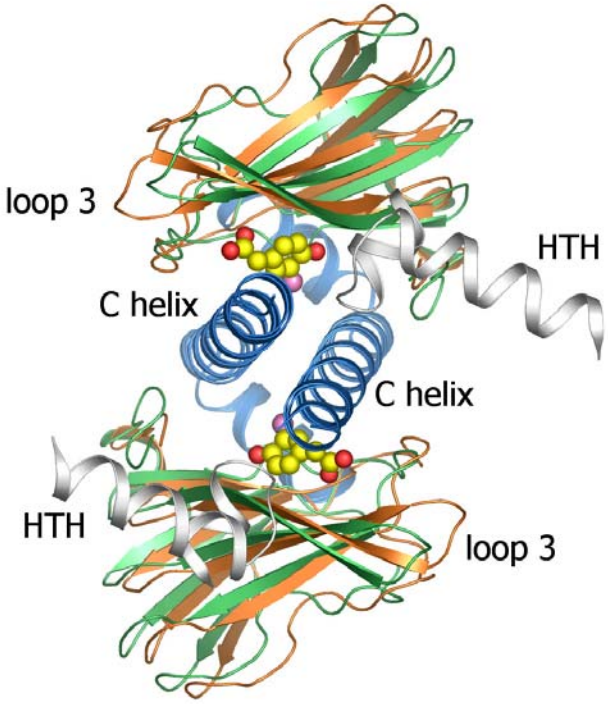

Figure 3.4 Overlay of the N-terminal domains of both CprK structures. An overlay created by superimposition of the B and C α-helices of both ligand-free CprK and the CHPA bound CprK structures with central helices coloured blue for both structures while the N-terminal β-barrel (residues 20-108 for both structures) is coloured green for the CHPA-CprK complex and orange for the ligand free CprK. Bound CHPA molecules in the CHPA-CprK structure are represented in atom coloured spheres. To illustrate the motion of the N-terminal β-barrel with respect to the putative position of the HTH motifs in the DNA-binding state (by analogy to CRP), the putative HTH motifs are represented in grey.

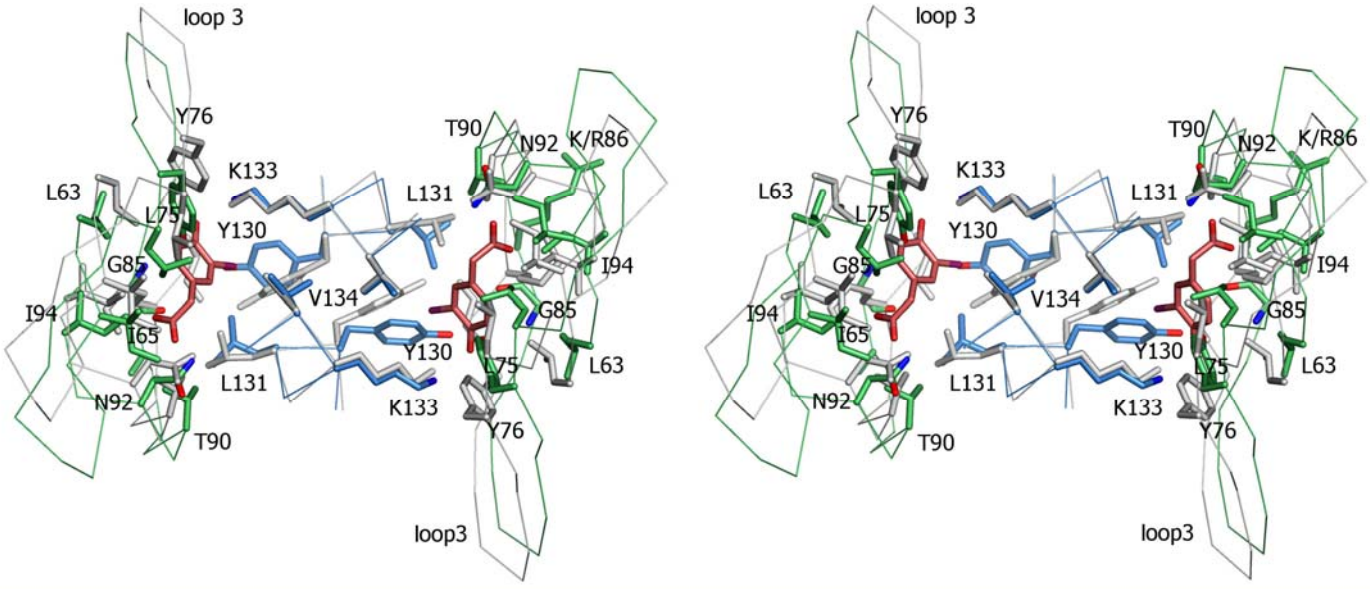

Figure 3.5 Overlay of the CHPA binding sites of both CprK structures. Using a similar superimposition as Fig. 3.4, the key binding site residues for both CprK structures are represented as in Fig. 3.3. The CHPA-CprK structure is coloured with grey carbon atoms (CHPA with purple carbon atoms), while the ligand-free CprK structure is represented with carbon atoms coloured according to Fig. 3.1.

Allosteric changes following binding of ortho-chlorophenolic compounds

Although binding of HPA, unlike CHPA, does not elicit a measurable allosteric transition, one can postulate that the local structure of the binding pocket in the HPA-CprK complex is similar to that in the CHPA-CprK complex, given the very high similarity between both compounds. However, as HPA does not induce any DNA binding, the overall structure of the HPA-CprK complex likely resembles that of ligand-free CprK. To model this putative CprK-HPA complex, the β-barrel of the ligand-bound structure was repositioned to occupy the conformation observed in the ligand-free structure. The resulting "hybrid" CprK structure thus contains local active site changes induced by ligand binding but the overall structure retains the unbound state (Fig. 3.7b). Then, overlaying this "hybrid" CprK structure on the ligand-free form reveals how ligand binding can induce, among other changes, a large shift in the position of Gly85 (Fig. 3.7a,b). The repositioning of Gly85 leads to formation of new hydrogen bonding interactions and direct steric contacts with the chloride atom binding residues Tyr130 and Leu131. This induces reorientation of Tyr130 and Leu131, thus reducing the volume of the hydrophobic pocket between the coiled coil helices to exactly match the volume of the chloride atom. These interactions made with CHPA/HPA and the "hybrid" CprK structure are very similar to those seen in the CHPA-CprK complex except that the phenol hydroxyl group is too distant from Lys133 to make a direct interaction. In addition, the chloride atom of CHPA is in unfavourably close contacts with Leu131 and Val134 as it is positioned slightly off-centre from the hydrophobic binding pocket. It seems likely that formation of the phenolate-Lys133 interaction and the correct positioning of the chloride atom within the hydrophobic pocket drive the rigid body motion of the N-terminal β-barrel (Figs. 3.3 and 3.7c).

While the pK_a for the phenol group of HPA is approximately ~10, this value is lowered to ~8.4 in CHPA due to the chloride substituent at the *ortho*-position (Spectrum Laboratories). We postulate that CprK can distinguish between CHPA and HPA not only on the basis of the additional steric bulk provided by the chloride atom, but also by a "pK_a interrogation" mechanism (Palfey *et al.*, 1999), which is based on the ability of ligands to ionize to the phenolate form and consequently interact with Lys133, driving the conformational change that promotes DNA binding. Among the several CprK homologues in the genome of *D. hafniense*, Lys133 is strictly conserved, which supports the "pK_a interrogation" concept. The other side chain interaction with the phenol hydroxyl group, Tyr76 is not conserved and the Tyr76Phe variant of *D. hafniense* CprK still undergoes allosteric changes upon binding of CHPA (albeit at 10-fold higher CHPA concentrations that the WT, Table 3.2), indicating this residue is not essential for activity. Most residues involved in binding the *ortho*-chlorophenol moiety are conserved or highly similar in CprK paralogs, while residues involved in binding the acetic acid group (e.g. Lys/Arg86, Thr90, Asn92) are specific to CprK, indicating that these CprK paralogues will likely interact with other compounds that have been found to serve as terminal electron acceptors such as 2,4-dichlorophenol and/or 2,4,6-trichlorophenol (Boyer *et al.*, 2003).

Conclusions

Crystal structures of CprK with and without CHPA reveal the binding mode for *ortho*-chlorophenols and suggest a mechanism whereby both the phenol hydroxyl group and the chloride *ortho*-substituent are required for tight binding and integrally involved in promoting the conformational change that leads to DNA binding. We postulate that binding of CHPA and concomitant phenol deprotonation leads to a rigid body hinge motion of the β-barrel that docks the chloride atom in the binding pocket provided by the central coiled coil and leads to formation of a tight phenolate-Lys133 interaction. While, in absence of a crystal structure for the reduced, CHPA bound CprK, it is difficult to determine the exact mechanism whereby the observed rigid body motions in the β-barrel are communicated to the DNA-binding domains, we postulate the sensory module reorganization repositions β strands 4 and 5 with respect to the central coiled coil and allows for a direct contact between the DNA-binding domain and the sensory module to be formed. This disrupts the DNA-binding domain dimer interface and positions the helix-turn-helix motifs in the required conformation for productive binding to the "dehalobox" DNA. The relative motion of the β-barrel to the B-C helix is reminiscent of similar motions postulated to occur in functionally unrelated proteins containing related sensory modules, such as cyclic nucleotide gated channels (CNG) and cAMP- and cGMP-dependent protein kinases (Kaupp *et al.*, 2002; Shabb *et al.*, 1992; Su *et al.*, 1995; Tibbs *et al.*, 1998; Varnum *et al.*, 1995). Formation of a disulphide bond under aerobic conditions between Cys11 and Cys200 does not impact CHPA binding but disrupts formation of the correct interdomain contacts and hence provides a possible means for redox regulation of halorespiration.

Acknowledgements

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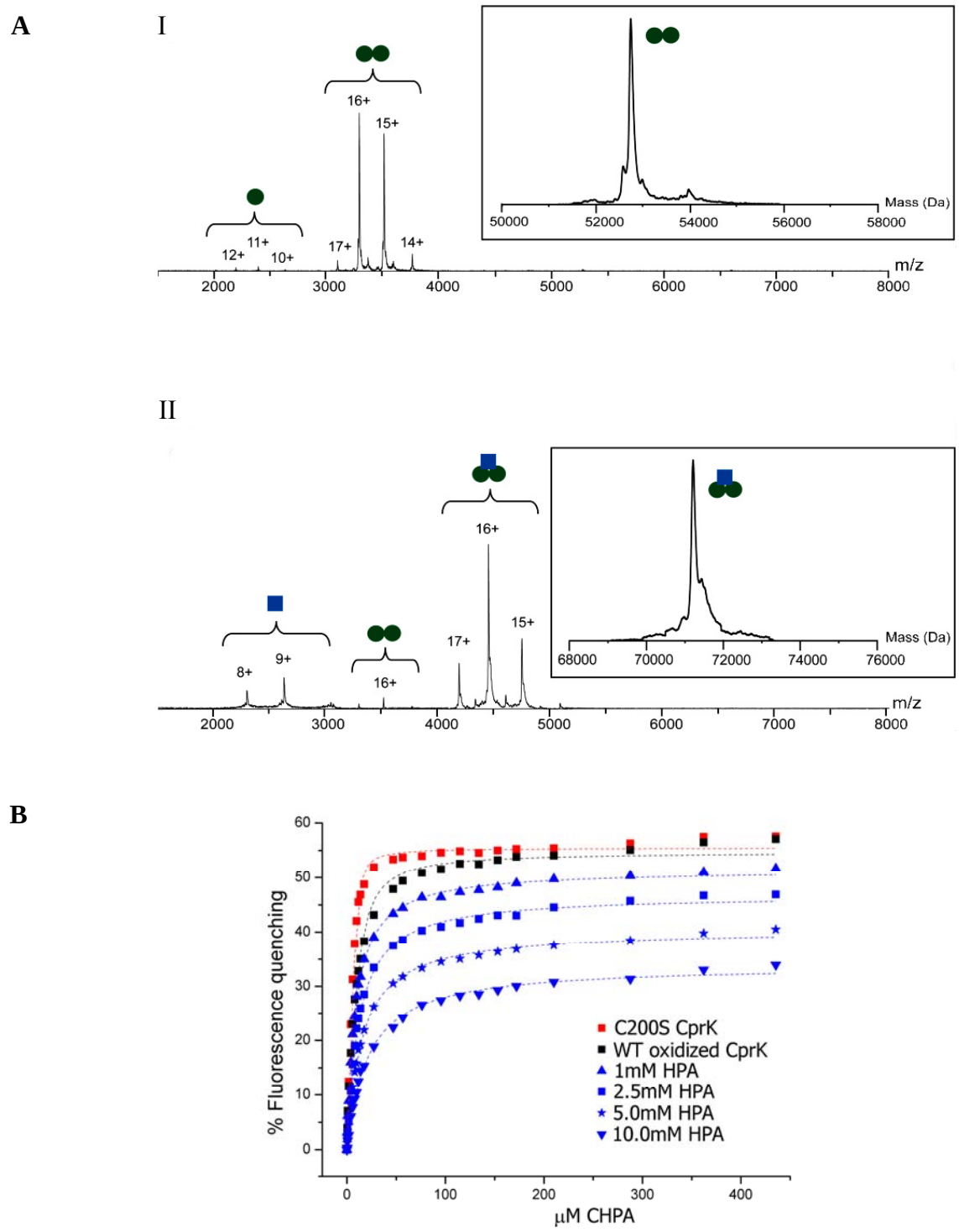

Figure 3.6 Solution data on *D. hafniense* CprK. (**A**) Native ESI-MS data. ESI-MS spectra of *D. hafniense* CprK sprayed from an aqueous 100 mM ammonium acetate solution [pH 8.0] at a dimer concentration of 4 μM in the presence of 10 mM dithiothreitol (I) and additionally in the presence of 5 μM dsDNA and 400 μM CHPA (II). The charges of the different ion series are indicated. Green circle and blue square indicate monomeric CprK and "dehalobox" DNA, respectively. The insets represent the deconvoluted mass spectra of dimeric CprK (I) and the complex between CprK and dsDNA (II). (**B**) Tryptophan fluorescence quenching obtained by titration of Cys200Ser *D. hafniense* CprK and oxidized wild type (WT) *D. hafniense* CprK with CHPA, in presence or absence of HPA. Data were fitted to a single exponential quadratic function.

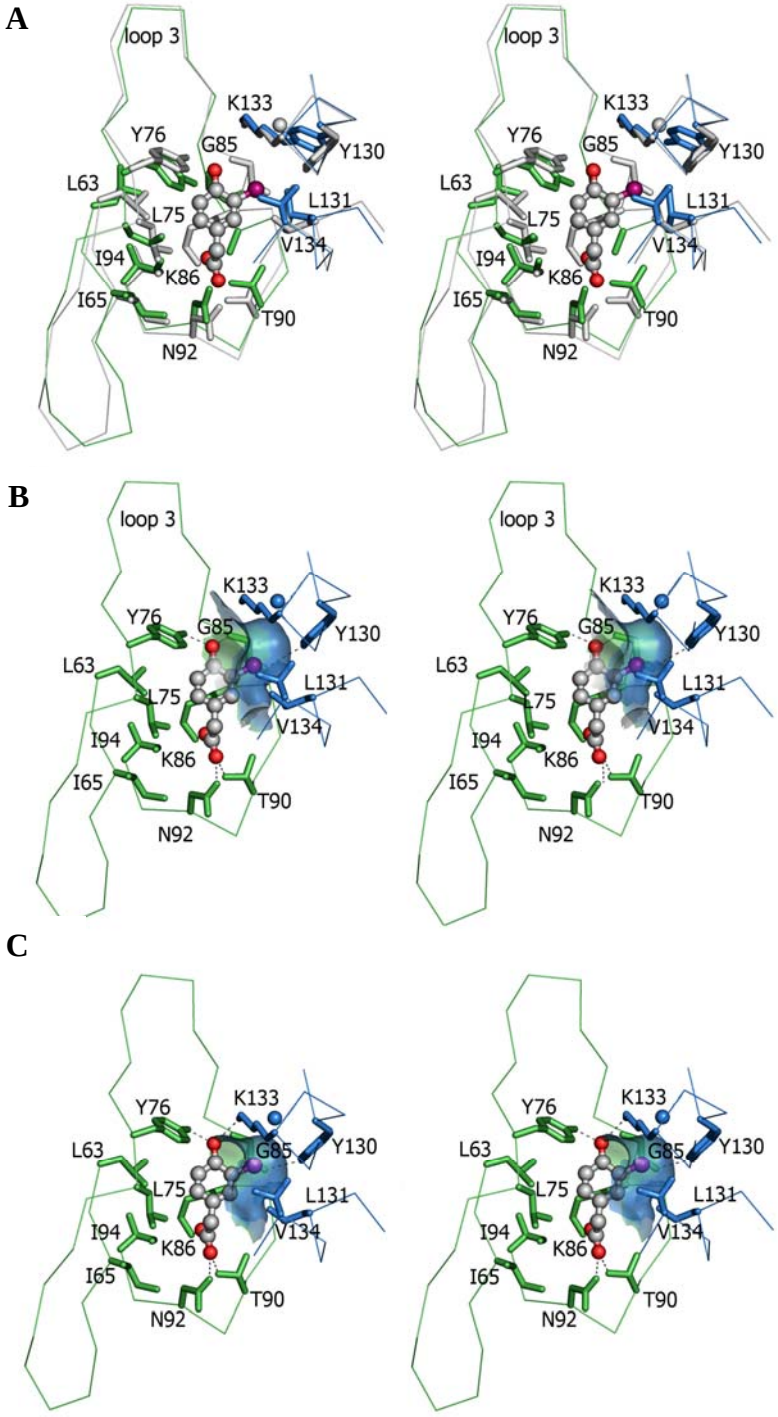

Figure 3.7 Binding of *ortho*-chlorophenolic compounds causes structural rearrangement. (**A**) Overlay of a single CHPA binding site of the ligand-free CprK (colour coding according to Fig. 3.1) with a "hybrid" CprK structure (in grey; see text). This clearly illustrates the induced fit in both the N-terminal β-barrel and the C-helix residues upon binding of CHPA. (**B**) Similar view to panel A, but only the "hybrid" structure is displayed, coloured coded as Fig. 3.1. The hydrophobic pocket created by the C-helix residues is depicted as a transparent surface. H-bonds between CHPA and CprK are depicted in dashed lines. No direct interaction can be made between CHPA and Lys133 while the CHPA chloride atom is not ideally placed in the binding pocket. (**C**) Similar view to B but for the CHPA-CprK crystal structure. The reorientation of the β-barrel has allowed for an additional interaction between CHPA and Lys133 while positioning the CHPA chloride atom in the centre of the hydrophobic cavity.

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4

Transcriptional activation by CprK1 is regulated by protein conformational changes induced by effector binding and reducing conditions

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submitted

Abstract

The transcriptional activator CprK1 from *Desulfitobacterium hafniense* is a homodimeric protein, which activates transcription of genes that encode proteins involved in reductive dehalogenation of chlorinated aromatic compounds. 3-Chloro-4-hydroxyphenylacetate is a known effector for CprK1, which interacts tightly with the protein, and induces binding to a specific DNA sequence ("dehalobox", TTAAT----ATTAA) located in the promoter region of chlorophenol reductive dehalogenase genes. Despite the availability of recent X-ray models of two CprK proteins in the reduced and oxidized state, the mechanism by which CprK1 activates transcription is poorly understood. Here, we determined the effector specificity of reduced CprK1 by using macromolecular native mass spectrometry. Only analogues of 3-chloro-4-hydroxyphenylacetate that have a halogen group at the *ortho* position and a chloride or acetic acid group at the *para* position were found to be potent effectors for CprK1, and thus induce binding to "dehalobox" DNA. By using limited proteolysis coupled to mass spectrometry, it has been demonstrated that CprK1 requires a cascade of structural events to interact with "dehalobox" DNA. Upon reduction of the intermolecular disulfide bridge in CprK1, the protein becomes more dynamic, but this alone is not sufficient to interact with "dehalobox" DNA. Triggering of CprK1 to its active state is a typical example of allosteric regulation: the binding of a potent effector molecule to reduced CprK1 induces local changes in the N-terminal effector-binding domain that are translated to the C-terminal DNA-binding domain by changes in inter-domain interactions inducing required conformational changes in the DNA-binding domain and subsequent transcriptional activation.

Introduction

Halogenated hydrocarbons are often toxic molecules that are widespread environmental pollutants because of their use in industry, agriculture and private households for example as pesticides, flame-retardants and degreasers. Although these compounds are generally very stable, they can be converted in many sediments and soils by reductive dehalogenation. Several strictly anaerobic bacteria capable of dehalogenation have been isolated including *Desulfomonile*, *Dehalobacter* and *Desulfitobacterium*. These organisms use chlorinated compounds as terminal electron acceptors (halorespiration), and thus remove the chloride atom while energy is conserved via electron-transport-phosphorylation (El Fantroussi *et al.*, 1998; Holliger *et al.*, 1998; Smidt & de Vos, 2004). The prospect of using these organisms in bioremediation of sediments contaminated with a variety of chlorinated aromatic compounds is promising. *Desulfitobacterium dehalogenans* can reductively dechlorinate phenolic compounds at the *ortho* position (Utkin *et al.*, 1995; Utkin *et al.*, 1994) and the closely related *Desulfitobacterium hafniense* DCB-2 (Christiansen *et al.*, 1996) can dechlorinate phenolic compounds at the *ortho* and *meta* position, the latter reaction, however, is described only for 3,5-dichlorophenol as a substrate (Madsen *et al.*, 1992).

A large number of the proteins involved in halorespiration are encoded in the chlorophenol reductive dehalogenase (*cpr*) gene cluster, which contains *cprTK$_1$ZEBA$_1$C* open reading frames in *D. hafniense*. The product of the *cprA1* gene, a 3-chloro-4-hydroxyphenylacetate (CHPA) reductive dehalogenase has been isolated and characterized from *D. hafniense* (Christiansen *et al.*, 1998). It was found that the *cprA1* gene is induced by the electron acceptor CHPA. The activation by CHPA is carried out at the transcription level by CprK1, a transcriptional activator protein that is encoded in the same *cpr* gene cluster. It was shown that CrpK1 from *D. hafniense* binds to a specific DNA sequence ("dehalobox", TTAAT----ATTAA), located in the promoter region of several *cpr* genes including *cprA1*, with high affinity in the presence of CHPA. This enables the recruitment of RNA polymerase, which then proceeds with transcription of the corresponding genes (Gábor *et al.*, 2006). It was also shown that CprK1 is redox-regulated and is active only in the reduced state. Previously, a close homologue called CprK was identified in the related halorespiring bacterium, *D. dehalogenans*, which shows 89% sequence identity with CprK1 from *D. hafniense.* Upon binding of CHPA, CprK activates transcription from dehalobox-containing promoters in *D. dehalogenans* (Pop *et al.*, 2004).

The primary sequence of CprK1 from *D. hafniense* contains 232 amino acids with a predicted molecular weight of 53 kDa for the dimeric protein. Recently, the X-ray structures of oxidized CprK1 from *D. hafniense* in complex with CHPA and reduced CprK from *D. dehalogenans* in its unliganded form have been determined by Joyce *et al.* (2006). The crystallographic models revealed that both CprKs exhibit high structural similarity to the cAMP receptor protein (CRP) of *Escherichia coli* (Fic *et al.*, 2006; Harman, 2001). CRP activates transcription of more than 100 genes in *E. coli* in the presence of the allosteric effector cAMP. In the X-ray model of CprK1 from *D. hafniense,* one CHPA molecule is bound per subunit in the β-barrel of the effector-binding domain. The observed binding site is highly similar to the binding site of cAMP in CRP (Joyce *et al.*, 2006). Two identical subunits of CprK1 form an asymmetric dimer with each monomer folded in two distinct domains: the N-terminal effector-binding domain and the C-terminal DNA-binding domain. The dimer interface of CprK1 is predominantly made up by the central α-helices connecting the sensory module with the DNA-binding domain containing a helix-turn-helix motif. In the model of the oxidized CprK1, an intermolecular disulfide bond connects the sensory module to the C-terminal DNA-binding helix of the opposite monomer. In the structure of unliganded and reduced CprK, the C-terminal DNA-binding domains dimerize in the non-DNA binding state. The comparison of the two crystal structures allowed to postulate that the binding of CHPA to the N-terminal β-barrels of CprK1 causes reorientation of the C-terminal DNA-binding domains with respect to the central α-helix at the dimer interface (Joyce *et al.*, 2006). The ultimate proof of this suggestion – i.e. the crystal structure of effector-bound reduced CprK1 protein – has not been achieved yet. The present X-ray models thus provide high-resolution structural information on CprK, however, they do not provide details about structural dynamics and do not allow the continuous monitoring of e.g. ligand- and/or DNA-induced conformational changes.

Besides the fact that structural changes are caused by the binding of an effector molecule, it is also important to find the crucial features that enable these molecules to cause such changes. Both CprKs bind CHPA with micromolar affinity promoting an interaction with "dehalobox" DNA (Joyce *et al.*, 2006). On the basis of the X-ray models it was suggested that i) the presence of the chloride group in the proper position and ii) the "pK_a interrogation" mechanism (formation of phenolate) allow DNA binding.

Here we report on the effector specificity of CprK1 and on the structural dynamics in CprK1 that are required for DNA binding. For this we used a small library of potential effector molecules known to be dehalogenated by *D. dehalogenans* and/or by *D. hafniense* (Table 4.1) in combination with macromolecular native mass spectrometry, limited proteolysis-mass spectrometry methods and electrophoretic mobility shift assays. In native mass spectrometry, the gentle nanoflow electrospray ionization technique is coupled to a time-of-flight mass spectrometer. This combination allows the direct observation of non-covalent complexes between proteins and between proteins and nucleic acids (Hanson *et al.*, 2004; Van den Heuvel *et al.*, 2005; Van den Heuvel *et al.*, 2004).

In this work we used the technique to study oligomerization of CprK1 and interaction with potential effectors and "dehalobox" DNA. Limited proteolysis coupled to mass spectrometry is a powerful method to probe the higher order structure of proteins (Hubbard, 1998) and to provide structural information of non-covalent interactions in solution by mapping protein-protein interfaces and protein-ligand interfaces in a medium-throughput fashion (Cohen *et al.*, 1995; Jamison *et al.*, 1994). Limited proteolysis-mass spectrometry allowed the characterization of the redox and effector-induced changes in dynamics or conformation in CprK1 that are required for transcriptional activation by this protein.

Materials and Methods

Preparation of CprK1 and "dehalobox" DNA

CprK1 was overproduced and purified according to a previously established method, using *Escherichia coli* BL21(DE3) and the pET24d-derived pWUR176 expression vector (Gábor *et al.*, 2006). CprK1 was analyzed by denaturing gel electrophoresis using a 10% (w/v) polyacrylamide gel. The gel was stained with 0.1% (v/v) Coomassie brilliant blue G250 for 3 min and destained overnight in 10% (v/v) acetic acid and 30% (v/v) methanol. Protein concentrations were determined using the method developed by Bradford (1976). The single stranded (ss) DNA 5'-AGGTAAAGTTAATACACATTAATACTTGCG-3' containing an inverted repeat ("dehalobox", underlined) and its complementary strand were synthesized by MWG (Ebersberg, Germany). Complementary oligonucleotides were annealed in a buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl and 1 mM EDTA by heating to 95°C and incubating at this temperature for 2 min then cooling down slowly to room temperature. The sample buffer was exchanged to ultrapure water using Vivaspin 0.5 ml 3,000 MW cut-off columns (Vivascience, Germany).

Electrophoretic mobility shift assay (EMSA)

A 52-bp DNA fragment from the promoter region of the *ortho*-chlorophenol reductive dehalogenase-encoding *cprA1* gene was PCR amplified from *D. hafniense* genomic DNA using oligonucleotide primers BG1748 (5'-GGTTGAGAAATTCAGGTAAAG-3') and BG1749 (5'-GGATCACATACGCAAGTATTAATG-3'). The resulting PCR product, which contained the TTAATacacATTAA CprK1-target sequence (dehalobox) was purified and radioactively labelled at the 5' end as described elsewhere (Gábor *et al.*, 2006). The EMSA reaction mixtures contained POP buffer (20% (v/v) glycerol, 50 mM Tris-HCl, pH 7.5 5 mM $MgCl_2$, 2.5 mM EDTA, 250 mM NaCl), 2.5 mM DTT, 1 µg poly(dG-dC)-poly(dG-dC), 1.5 nM ^{32}P-labelled DNA and 0.25 µM purified CprK1 (dimer concentration). Effector compounds, if present, were added from an aqueous stock solution to a final concentration of 6.25 µM. After incubation at 24°C for 30 min, the obtained protein-DNA complexes were separated on a non-denaturing 6% (w/v) polyacrylamide gel and electrophoresed at 10 mA constant current at 4°C in TB buffer (89 mM Tris and 89 mM boric acid). The gels were then dried, exposed to a phosphor screen for several hours and analyzed.

Macromolecular native mass spectrometry

For nanoflow electrospray mass spectrometry experiments, ultrafiltration units (ultrafree-0.5 Centrifugal Filter Device, Millipore, USA) with a cut-off of 5,000 Da were used to exchange the buffer of CprK1 sample in 150 mM ammonium acetate pH 6.8 or in 100 mM ammonium acetate pH 8.0. For DNA binding measurements, 5-10 mM dithiothreitol, 40-400 µM phenol derivative, 2-5 µM dsDNA and 4 µM Cprk1 (dimer concentration) were mixed and incubated for 30 min at room temperature. The molar ratio between dimeric CprK1 and dsDNA was 2:1 for pH 6.8 and 1:1.3 for pH 8.0. The phenol derivatives (CHPA, HPA, 2,4-DCP, 2-Br-4-CP, 2,3-DCP, 3,5-DCP, 2,4,5-TCP, and 2,4,6-TCP) were dissolved in water or in 100 mM ammonium acetate pH 8.0. Native mass spectrometry measurements were performed in positive ion mode using an electrospray ionization time-of-flight instrument (LC-T; Micromass, UK) equipped with a Z-spray nano-electrospray ionization source. In order to produce intact ions *in vacuo* from large complexes in solution, ions were cooled by increasing the pressure in the first vacuum stages of the mass spectrometer (Krutchinsky *et al.*, 1998; Tahallah *et al.*, 2001). In addition, efficient desolvation was needed to sharpen the ion signals in order to determine the stoichiometry of the complexes from the mass spectrum. Therefore, source pressure conditions and electrospray voltages were optimized for transmission of the macromolecular protein complexes. The needle and sample cone voltage were 1,400 V and 140 V, respectively. The pressure in the interface region was adjusted to 8 millibar by reducing the pumping capacity of the rotary pump by closing the speedivalve. Electrospray needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments, USA) on a P-97 puller (Sutter Instruments, USA), coated with a thin gold layer by using an Edwards Scancoat (Edwards Laboratories, USA) six Pirani 501 sputter coater. The spectra were mass calibrated by using a solution of 10 mg/ml cesium iodide in 50% (v/v) isopropanol.

Limited proteolysis coupled to matrix assisted laser desorption/ionization mass spectrometry

For limited proteolysis measurements 6 μM CprK1 (dimer concentration) was incubated in the absence or presence of 400 μM effector, 29 μM "dehalobox" DNA and 10 mM dithiothreitol in 100 mM ammonium acetate pH 8.0 for 30 min at room temperature. Limited proteolysis of CprK1 was performed using sequencing grade modified (0.27 μM) trypsin (Roche, The Netherlands). Aliquots of 2 μl of each digest were taken at 2 min, 15 min, 1 hr, 6 hrs, and 22 hrs, and mixed with 8 μl of 0.2% (v/v) trifluoroacetic acid to quench trypsin activity. Trypsin digests were desalted and concentrated with μC18-ZipTips (Millipore, USA) and analyzed on a matrix assisted laser desorption/ionization ToF-ToF mass spectrometer (Applied Biosystems 4700 Proteomics analyzer, USA) using α-cyano-4-hydroxycinnamic acid as the matrix. The mass spectra were recorded in positive ion reflectron mode at 20 kV accelerating voltage and spectra were internally calibrated using the single protonated trypsin autodigestion peaks. The raw mass spectra were processed using Data Explorer software, version 4.0.

Fluorescence measurements

Fluorescence experiments were performed with an SPF 500c spectrofluorimeter (SLM Aminco, USA) at 20°C. Excitation was carried out at 280 nm and emission fluorescence was recorded at 330 nm. A 10-mm path length quartz cell with a stirrer was used. Excitation and emission slit widths were 10 and 5 nm, respectively. Solutions contained 0.2 μM CprK1 (dimer concentration) and 0.75 mM dithiotereitol in 100 mM ammonium acetate, pH 8.0. Upon mixing the phenolic compound and CprK1, fluorescence quenching was monitored in time. The data were analyzed by means of the equation $F = F1*[L_t]/(K_D+[L_t])$, where F is the fluorescence emission at 330 nm at a given added ligand concentration, F1 the fluorescence emission at the end of the titration, K_D the apparent dissociation constant, and $[L_t]$ the total ligand concentration.

Results

The quaternary structure of CprK1 does not depend on the protein redox state

Previous studies have revealed that CprK1 is capable of binding "dehalobox" DNA only in its reduced state and that its DNA-binding activity is inhibited by the thiol-oxidizing agent diamide (Gábor *et al.*, 2006; Pop *et al.*, 2004). Oxidized CprK1 contains an intermolecular disulfide bond between Cys11 of monomer A and Cys200 of monomer B and *vice versa* (Joyce *et al.*, 2006). We performed denaturing gel electrophoresis of CprK1 under oxidizing and reducing conditions and different pH values to determine the conditions necessary to reduce CprK1 *in vitro* (Fig. 4.1a). The data clearly showed that CprK1 (pI 8.1) was almost fully reduced at pH 8 at dithiothreitol concentrations of 5 and 10 mM, but remained oxidized to some extent at pH 6.8.

We studied the oligomerization state of CprK1 at pH 8 under reducing and oxidizing conditions by using native nanoflow electrospray ionization mass spectrometry (Fig. 4.1b). In both conditions, one dominant ion series was observed around 3,400 mass-to-charge (m/z) values corresponding to a mass of 52,739 ± 4 Da, representing dimeric CprK1. The measured mass of the dimeric species was lower than the expected mass of the dimer (52,998 Da) on the basis of the primary amino acid sequence, which is due to the deletion of the the N-terminal methionine in CprK1. In both mass spectra we also observed a very low abundant species with a mass of 53,003 ± 7 Da, which is the full-length dimeric protein, thus including the N-terminal methionine. These data thus indicate that the redox state of CprK1 does not influence its oligomeric state.

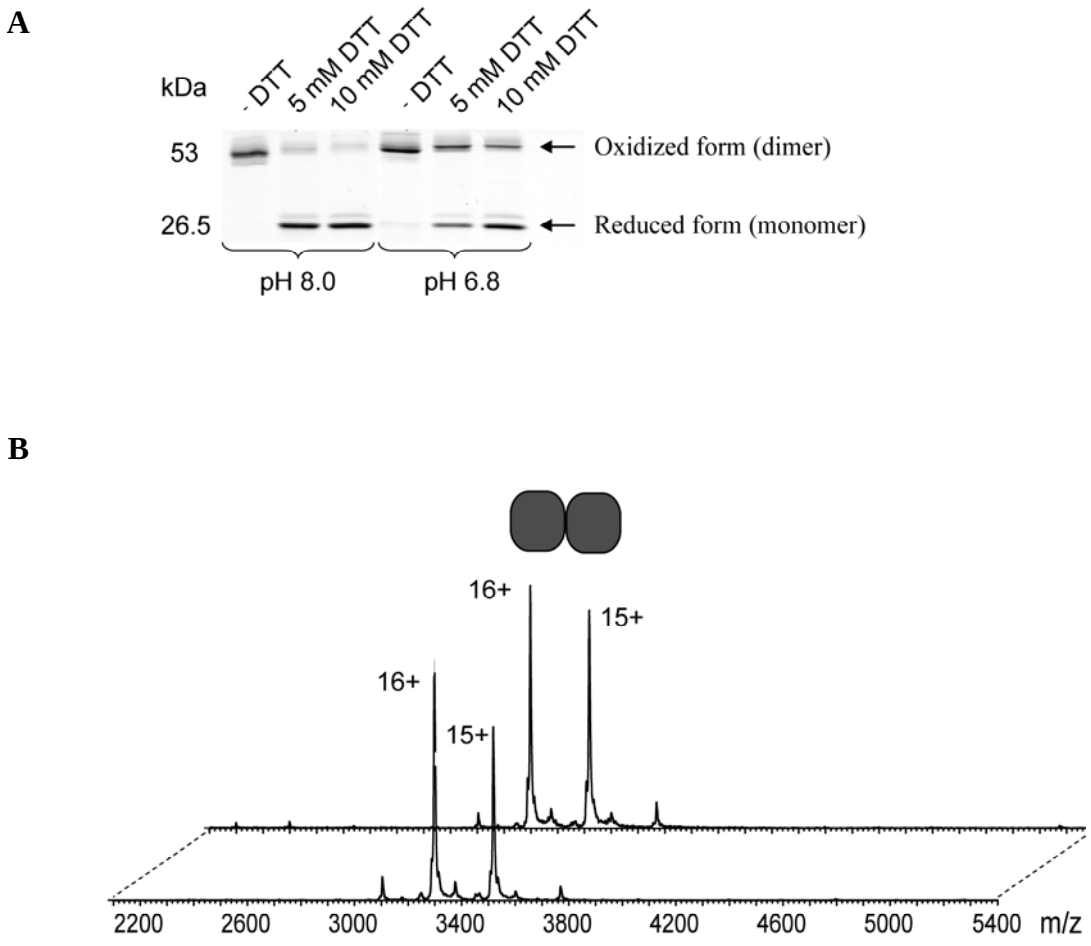


Figure 4.1 The quaternary structure of CprK1 is independent on the protein redox state. (**A**) Denaturing polyacrylamide gel of 4 μM CprK1 incubated in the presence or absence of 5 or 10 mM dithiothreitol in a 150 mM aqueous ammonium acetate solution, pH 6.8 or 100 mM aqueous ammonium acetate, pH 8. (**B**) Electrospray ionization mass spectra of CprK1 sprayed from an aqueous 100 mM ammonium acetate solution, pH 8 at a protein dimer concentration of 4 μM in the absence or presence of 10 mM dithiothreitol. Rectangles represent dimeric CprK1 species.

CprK1-mediated DNA binding is induced by reducing conditions and the effector CHPA

The phenol derivative CHPA is a known effector of CprK1 (Gábor *et al.*, 2006). Recently, we have demonstrated by native mass spectrometry that this compound induces binding of CprK1 to "dehalobox" DNA and that dimeric CprK1 interacts with one molecule of DNA at pH 8 (Joyce *et al.*, 2006). Figure 4.2a shows the electrospray ionization mass spectrum of CprK1 incubated with "dehalobox" DNA at pH 8 under reducing conditions. In this mass spectrum we observed three ion series around m/z 2,500, 3,400 and 4,400, respectively. The first series represents "dehalobox" DNA with a determined mass of 18,410 ± 3 Da (expected mass 18,407 Da). The second and third series represent dimeric CprK1 and the complex between dimeric CprK1 and one molecule of DNA with a determined mass of 71,165 ± 3 Da (expected mass 71,143 Da), respectively. The observed slight mass increase is likely due to water or buffer molecules still present in the CprK1-DNA complex (Sobott *et al.*, 2005).

Figure 4.2b shows the mass spectrum obtained after the addition of 400 μM CHPA to the "dehalobox" DNA-CprK1 solution at pH 8 under reducing conditions. The spectrum clearly shows a transition from free CprK1 and free DNA to a CprK1-DNA complex. CHPA did not induce DNA binding if the experiment at pH 8 was repeated under oxidizing conditions (Fig. 4.2c), confirming the importance of the reduction of the disulfide bridge. We did not observe interaction of CprK1 with 30-mer dsDNA having a random sequence (data not shown), strongly indicating that the interactions are specific for the DNA sequence containing the TTAAT----ATTAA dehalobox.

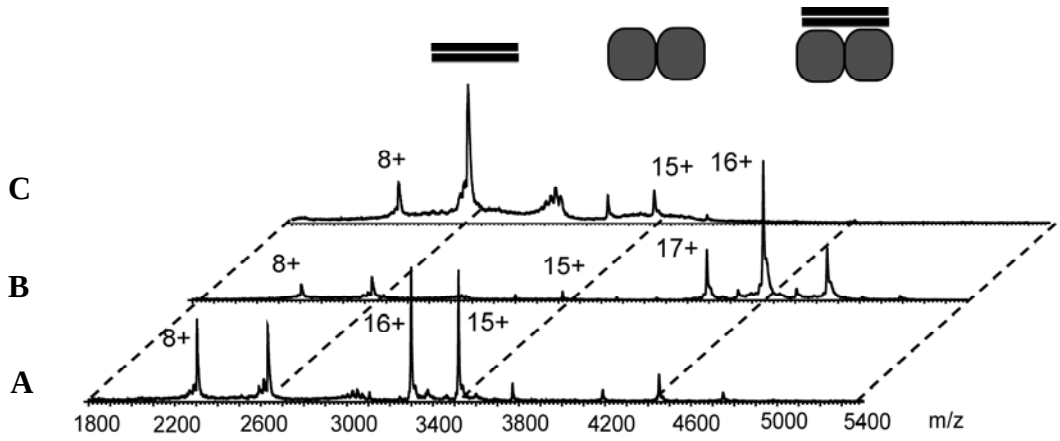


Figure 4.2 CprK1 interacts tightly with "dehalobox" DNA under reducing conditions and in the presence of CHPA. Electrospray ionization mass spectra of CprK1 sprayed from an aqueous 100 mM ammonium acetate solution pH 8 at a protein dimer concentration of 4 μM in the presence of 5 μM 30-bp "dehalobox" DNA under different conditions: (**A**) in the presence of 10 mM dithiothreitol, (**B**) in the presence of 10 mM dithiothreitol and 400 μM CHPA and (**C**) under oxidizing conditions and in the presence of 400 μM CHPA. Rectangles correspond to the dimeric CprK1, the double lines to dsDNA.

Intriguingly, although the addition of CHPA enhanced efficiently the binding of DNA to CprK1, the ternary complex comprising CHPA was not observed. CHPA interacts tightly with CprK1 (0.83 μM) and the complex is also retained in the CprK1 crystals (Joyce *et al.*, 2006). Thus, in solution-phase a strong interaction exists between CprK1 and CHPA, but this complex dissociates in the process of forming gas-phase ions or during the analysis of gas-phase ions in the mass spectrometer. It should be noted here that the applicability of mass spectrometry to investigate non-covalent biological complexes is strongly dependent on the nature of the interactions between the partners in the complex (Rogniaux *et al.*, 2001). When hydrophobic interactions play a prominent role in the binding, the preservation of the complex during the electrospray measurements is highly compromised (Robinson *et al.*, 1996). Indeed, the crystallographic data show an important role of hydrophobic interactions in the binding between CprK1 and CHPA, however, there are also six hydrogen bond interactions (Y76, G85, K86, T90, N92 and Y130) and two salt bridges (K86 and K133) between the protein and CHPA (Joyce *et al.*, 2006).

CprK1 is activated by different phenolic compounds

The *ortho*-chlorophenol CHPA is a strong effector for CprK1, however, the identity of alternative effector molecules has not been studied previously. Here, we investigated the specificity of CprK1 by using a library of potential effector molecules (Fig. 4.3). CprK1 was mixed with "dehalobox" DNA and different concentrations of phenolic compound at pH 8 under reducing conditions. The effector potential of the different phenolic compounds was determined by measuring the relative amount of DNA-CprK1 complex formed upon incubation with the specific compound using native mass spectrometry (Table 4.1, Fig. 4.4a,b). The data clearly show that both CprK1 reduction and effector binding are required for efficient "dehalobox" DNA binding. Under reducing conditions, but in the absence of a phenolic compound, no complex was formed between CprK1 and the "dehalobox" DNA. Similar results were obtained when reduced CprK1 was incubated with 4-hydroxyphenylacetate (HPA), a phenol derivative lacking a halogen atom at the *ortho* position, cAMP or with the *meta*-substituted 3,5-dichlorophenol (3,5-DCP). These three compounds are not effectors of CprK1 (class I). The compound 2,3-dichlorophenol (2,3-DCP) is a moderate effector, which at a high molar excess exhibits an allosteric effect on CprK1 by promoting protein-DNA complex formation (class II). CHPA, 2,4-dichlorophenol (2,4-DCP), 2-bromo-4-chlorophenol (2-Br-4-CP), 2,4,5-trichlorophenol (2,4,5-TCP) and 2,4,6-trichlorophenol (2,4,6-TCP) are strong effectors and thus have a strong allosteric effect on CprK1 (class III). The fact that we did not observe 100% complexation between CprK1 and DNA even in the presence of strong effectors is likely caused by incomplete reduction of the protein meaning that a low amount of oxidized protein was still present in the protein solutions (Fig. 4.1a).

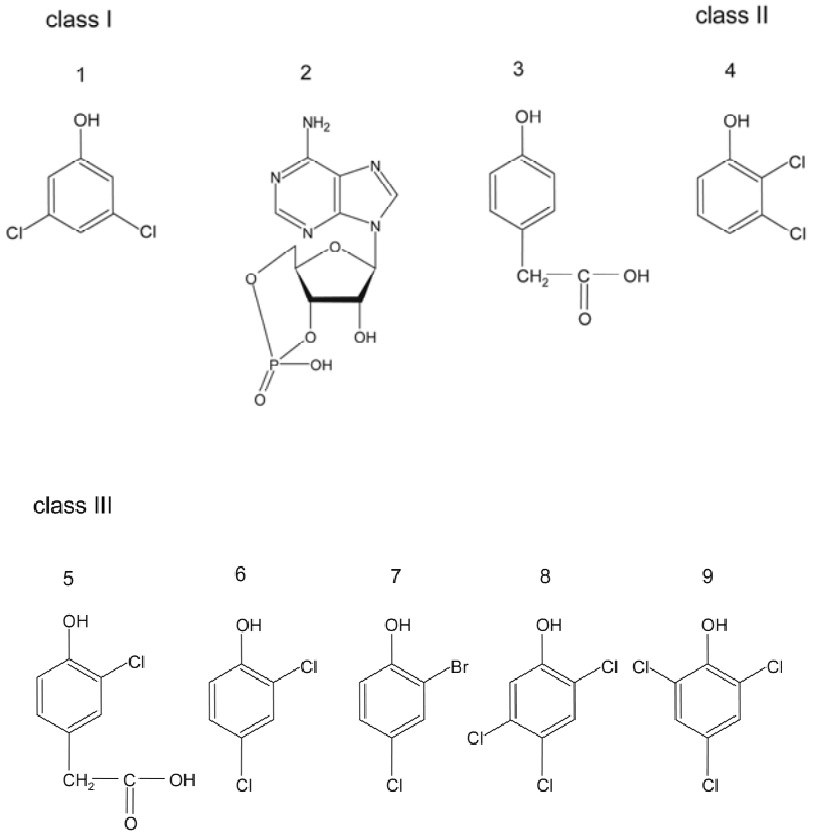


Figure 4.3 The molecular structures of the library of potential effectors for CprK1. The molecules are classified in three classes with increasing effector activity. Class I: 3,5-dichlorophenol (3,5-DCP) (1), cAMP (2) and 4-hydroxyphenylacetic acid (HPA) (3), class II: 2,3-dichlorophenol (2,3-DCP) (4) and class III: 3-chloro-4-hydroxyphenylacetic acid (CHPA) (5), 2,4-diclorophenol (2,4-DCP) (6), 2-bromo-4-chlorophenol (2-Br-4-CP) (7), 2,4,5-trichlorophenol (2,4,5-TCP) (8) and 2,4,6-trichlorophenol (2,4,6-TCP) (9).

Electrophoretic mobility shift assays (EMSA) were used to validate the data that were obtained by native mass spectrometry. CprK1 was incubated with radioactively labelled "dehalobox" DNA in the presence of potential effector molecules. The protein-DNA complexes were resolved by applying them on a non-denaturing gel. The results obtained for the interaction between DNA and protein in solution corresponded well with the native mass spectrometry data (Table 4.1, Fig. 4.4c). In the absence of effector, or in the presence of HPA, 3,5-DCP, 2,3-DCP or cAMP no protein-DNA complexes were formed. On the other hand 2,4-DCP, 2-Br-4-CP, 2,4,5-TCP and CHPA had an allosteric effect on CprK1 by promoting protein-DNA complex formation. The most prominent allosteric effect was observed for CHPA. Differently from the native mass spectrometry data, the presence of 2,4,6-TCP did not induce "dehalobox" DNA binding to reduced CprK1 under the conditions used in EMSA, whereas this compound was classified as a strong effector in the mass spectrometry studies. The reason for this remains an open question.

The dissociation constants of the different phenol-derivatives were determined by fluorescence quenching measurements (titration curves are presented in Supplementary figure 4.1) under reducing conditions (Table 4.1). We measured a dissociation constant of 1.4 μM for CHPA, which is in agreement with the value (0.83 μM) measured previously by Joyce *et al.* (2006). All other strong effectors have dissociation constants less than 30 μM, whereas the moderate effectors and the compounds that are not effectors have dissociation constants of more than 200 μM. These data clearly show that binding affinity and effector role are closely correlated.

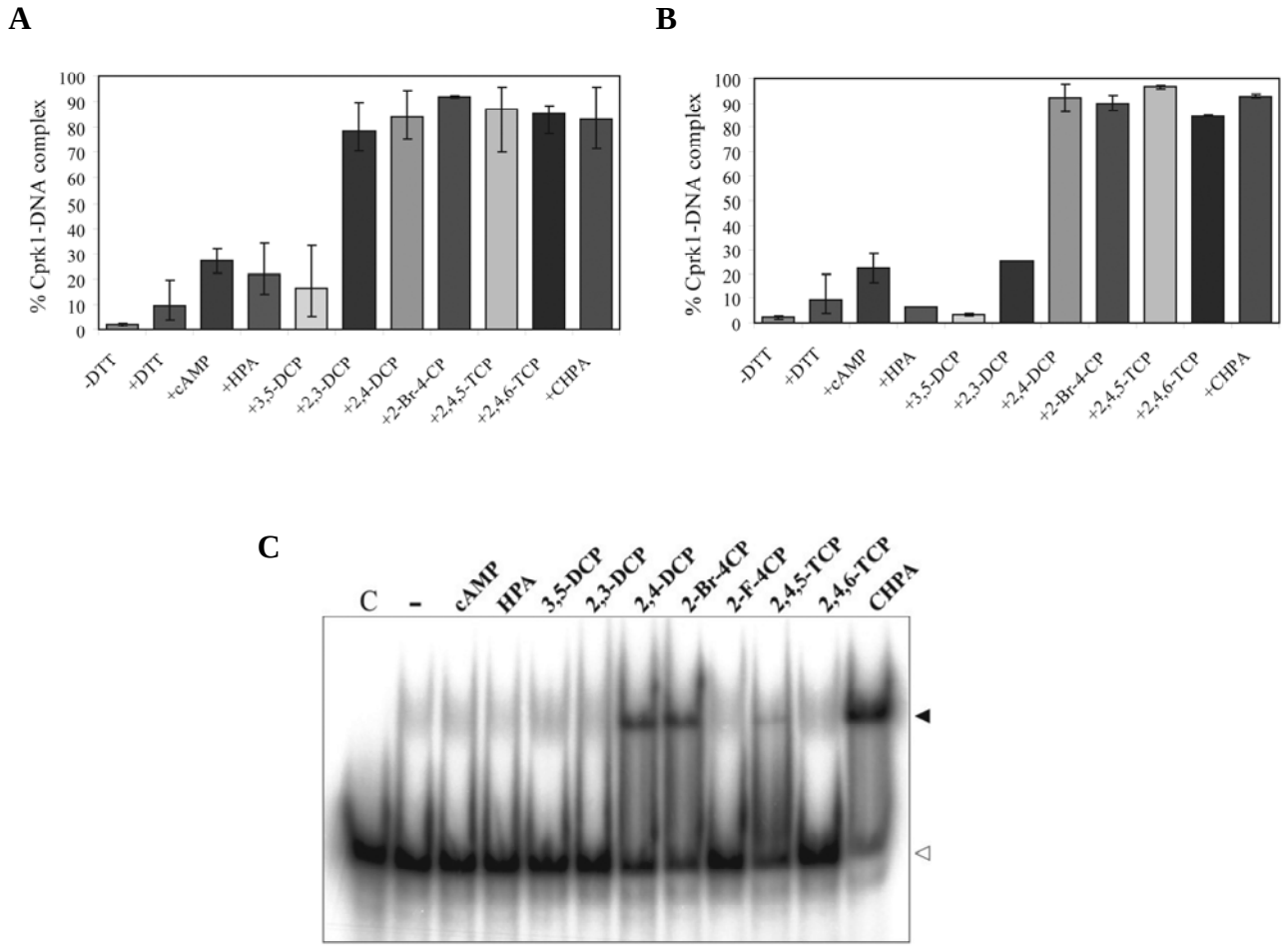


Figure 4.4 The effector specificity of CprK1. Panel (**A**) and (**B**) show diagrams of electrospray ionization mass spectra of CprK1 sprayed from an aqueous 100 mM ammonium acetate solution, pH 8 at a protein dimer concentration of 4 μM and incubated in the presence of 10 mM dithiothreitol, 5 μM 30-bp "dehalobox' DNA and 400 μM (**A**) or 40 μM (**B**) of a potential effector molecule. The diagrams present the percentage of CprK1-"dehalobox" DNA complex formed relative to the cumulative intensity of CprK1 and CprK1-DNA complexes. (**C**) Electrophoretic mobility shift assays from mixtures of CprK1 and a 52-bp "dehalobox" DNA. Binding mixtures contained no protein (lane C) or 0.25 μM CprK1 (dimer concentration) and 6.25 μM effector molecules as indicated above the gel. Free "dehalobox" DNA (fast migrating band) and CprK1-DNA complexes (band with retarded electrophoretic mobility) are indicated with an open or filled arrowhead, respectively.

Table 4.1 Potential effector molecules of CprK1 from *D. hafniense*. The dissociation constants of the different molecules were determined by tryptophan fluorescence. Electrospray ionization mass spectrometry and electrophoretic mobility shift assays (EMSA) were used to determine the allosteric effect of the molecules on CprK1 by promoting DNA complex formation.

Compounds	pK_a	Dissociation constant (μM)	Mass spectrometry	EMSA
CHPA	8.3	1.4	Strong effector	Effector
2,4-DCP	7.7	20.0	Strong effector	Effector
2-Br-4-CP	7.7	29.3	Strong effector	Effector
2,4,5-TCP	6.9	9.9	Strong effector	Effector
2,4,6-TCP	6.0	29.9	Strong effector	Not effector
2,3-DCP	7.4	207.0	Moderate effector	Not effector
3,5-DCP	8.3	259.4	Not effector	Not effector
HPA	10.1	6200[a]	Not effector	Not effector
cAMP	not measured	not measured	Not effector	Not effector

[a] Dissociation constant as determined by Joyce *et al.* (2006)

The structural dynamics in CprK1 is regulated by the protein redox state

The results described above (Fig. 4.2) and previous studies have shown that only reduced CprK1 can be activated by an effector compound (Gábor *et al.*, 2006; Pop *et al.*, 2004). As the disulfide bridge between Cys11 and Cys200 is disrupted upon reduction, conformational changes in protein dynamics might be of importance for the activation process. We investigated the dynamics and/or conformational changes by using a combination of limited proteolysis and mass spectrometry, using the specific proteases trypsin or endoproteinase Glu-C to partially digest CprK1. Trypsin cleaves proteins at the carboxyl side of the basic amino acids lysine and arginine, except when these two residues are followed by proline, while Glu-C cleaves after glutamate residues (under the conditions we used), unless they are followed by proline. We monitored the limited proteolysis by taking aliquots at various time-points (2 min, 15 min, 1 hr, 6 hrs and 22 hrs), and analyzed the reaction mixture by matrix assisted laser desorption/ionization mass spectrometry (see Supplementary figures 4.2-4.5 for spectra). First, we investigated the dynamics and/or conformational changes of CprK1 under reducing and oxidizing conditions in the absence of effector and "dehalobox" DNA. Figure 4.5 presents the amino acid sequence of CprK1 and shows the identified peptides after limited proteolysis. The sequence coverage after 22 hrs of incubation of CprK1 was 91.3% and 56.7% with trypsin and endoproteinase Glu-C, respectively. The observed proteolytic peptides are presented in Table 4.2.

Under oxidizing conditions, the peptides T2-T21 and T4-T21 were observed after 1 hr reaction time, confirming the presence of a disulfide bridge between Cys11 and Cys200 (Table 4.2 and Supplementary figure 4.2c). These two peptides were not detected under reducing conditions. In contrast, the peptides T1, T2, T3, T4 and T21, uniquely formed when the disulfide bridge is reduced, were observed after 2 or 15 min reaction time. These non-disulfide bridged peptides appeared at an earlier time point compared to the corresponding disulfide bridged peptides (1 hr for T2-T21 and T4-T21) strongly indicating that this region of CprK1 is more dynamic under reducing conditions.

Under oxidizing conditions, nine rapid cleavages (2 and 15 min) were observed, which are located at the surface of the protein, therefore accessible to the solvent (Table 4.2, Fig. 4.6a). These cleavage sites were divided over the N-terminal effector-binding domain (residues 1-144) and the C-terminal DNA-binding domain (residues 145-232). The residues R26, R35, K39 located in the effector-binding domain appeared at 15 min, K166, R167, K181, R196, and K223 located in the DNA-binding domain appeared also at 15 min, but K213 corresponding to the largest C-terminal trypsin peptide appeared already at 2 min. After 1 hr, 6 hrs and 22 hrs of incubation seven, six and one additional cleavage sites were observed, respectively.

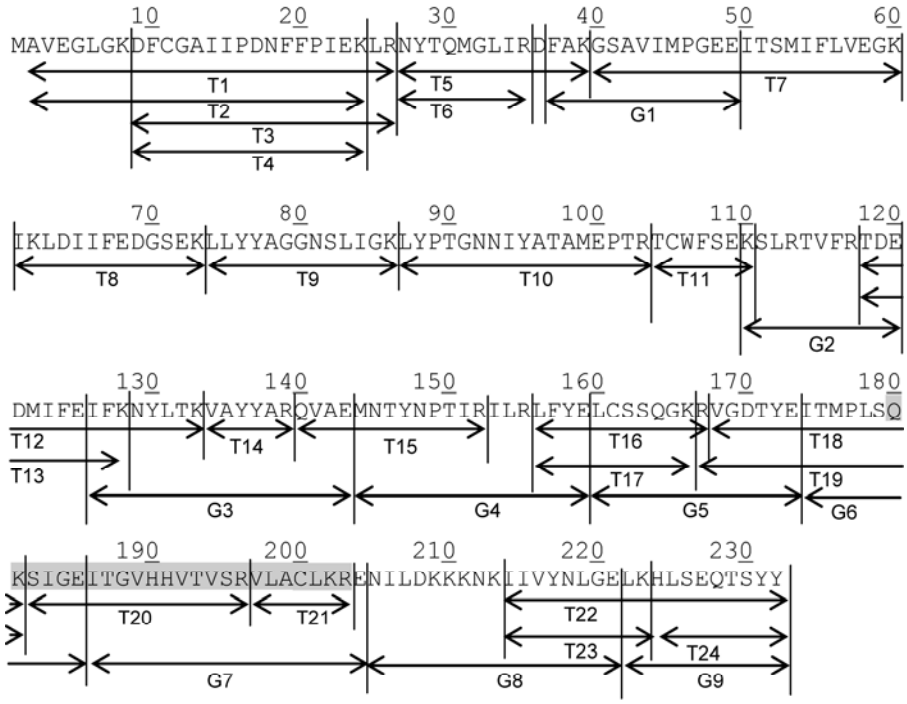


Figure 4.5 Peptides identified after limited proteolysis of native CprK1. Horizontal arrows indicate the cleavage products after enzymatic digestion using trypsin (T) or endoproteinase Glu-C (G). Highlighted residues indicate the DNA-binding helix-turn-helix motif. The sequence coverage after 22 hrs of incubation under native conditions was 91.3% and 56.7% with trypsin and endoproteinase Glu-C, respectively.

Table 4.2 Reaction time for appearance of peptide bond cleavage of oxidized CprK1, reduced CprK1, reduced CprK1 in presence of CHPA, and reduced CprK1 in presence of CHPA and "dehalobox" DNA.

Reaction time for appearance of peptide bond cleavage					
Peptide bond[a]	**Identified peptides[b]**	**Oxidized CprK1**	**Reduced CprK1**	**Reduced CprK1 + CHPA**	**Reduced CprK1 + CHPA + DNA**
K^{8}-D^{9}	T3, T4	nd	15 min	nd	nd
K^{8}-D^{9}	T4-T21[c]	1 h	nd	nd	nd
K^{24}-L^{25}	T2, T3, T4	nd	15 min	nd	nd
K^{24}-L^{25}	T4-T21[c]	1 h	nd	nd	nd
R^{26}-N^{27}	T1	nd	2 min	nd	nd
R^{26}-N^{27}	T5	15 min	2 min	nd	nd
R^{35}-D^{36}	T6	15 min	15 min	nd	nd
K^{39}-G^{40}	T5	15 min	2 min	nd	nd
K^{60}-I^{61}	T7, T8	6 h	22 h	nd	nd
K^{73}-L^{74}	T9	6 h	22 h	nd	nd
K^{86}-L^{87}	T10	1 h	15 min	22 h	22 h
R^{103}-T^{104}	T10, T11	6 h	15 min	22 h	22 h
K^{110}-S^{111}	T11	6 h	1 h	nd	nd
R^{117}-T^{118}	T12, T13	6 h	22 h	nd	nd
K^{128}-N^{129}	T13	6 h	22 h	nd	nd
K^{133}-V^{134}	T14	1 h	1 h	nd	nd
R^{139}-Q^{140}	T14, T15	1 h	1 h	nd	nd
R^{152}-I^{153}	T15	1 h	1 h	nd	nd
R^{155}-L^{156}	T16, T17	22 h	15 min	15 min	15 min
K^{166}-R^{167}	T19	15 min	15 min	15 min	15 min
R^{167}-V^{168}	T18	15 min	15 min	15 min	15 min
K^{181}-S^{182}	T20	15 min	2 min	2 min	2 min (low level)
R^{196}-V^{197}	T20	15 min	2 min	2 min	2 min (low level)
R^{196}-V^{197}	T21	nd	15 min	15 min	15 min
R^{196}-V^{197}	T2-T21[c]	1 h	nd	nd	nd
R^{203}-E^{204}	T21	nd	15 min	15 min	15 min
R^{203}-E^{204}	T2-T21[c]	1 h	nd	nd	nd
K^{213}-I^{214}	T22	2 min	2 min	2 min	2 min
K^{223}-H^{225}	T23, T24	15 min	15 min	15 min	15 min

[a] Amino acid numbers refer to the primary amino acid sequence of CprK1; [b] Digest fragment nomenclature refers to CprK1 protein; [c] Disulfide bridged peptide; nd, Not detected at 22 hrs

They correspond to the α-helix of the monomer-monomer interface (R117, K128, K133, R139), and to the effector-binding domain including the buried β-barrel (K60, K73, K86, R103, K110). The cleavage sites K8, K24 and R203 were also protected due to the disulfide bridge as we showed above. Finally, two sites that are located in the N-terminal α-helix of the DNA-binding domain showed a protection: R152 appeared after 1 h digestion, while R155 could only be detected after 22 hrs digestion.

Under reducing conditions, most of the cleavage sites located at the surface of the protein were more accessible to the trypsin digestion (Table 4.2, Fig. 4.6b). The same nine rapid cleavages sites were observed but four instead of one appeared already at 2 min (R26, K39, K181, and R196), showing an increased flexibility of the protein due to the reduction of the disulfide bridge. We observed six other rapid cleavage sites (K8, K24, K86, R103, R155 and R203) at 15 min, including a site (R155) from the DNA-binding domain which was almost fully protected from digestion under oxidizing conditions. Overall, the data show that under reducing conditions CprK1 becomes globally more flexible (Figs. 4.6a and 4.6b). However, four cleavage sites (K60, K73, R117 and K128) showed an opposite trend; they appeared earlier in the oxidized enzyme than in the reduced enzyme, indicating that reducing conditions can locally induce a more rigid quaternary CprK1 structure. The residues R117 and K128 belong to the α-helix of the monomer-monomer interface, thus their protection indicates tighter interaction between the two monomers under reducing conditions.

Effector and "dehalobox" DNA binding induce conformational and structural dynamics changes in reduced CprK1

In order to study the conformational effects of effector and "dehalobox" DNA binding to CprK1, limited proteolysis experiments using trypsin were performed under reducing conditions, in the presence of the different effectors (Fig. 4.3) and "dehalobox" DNA. Two effector concentrations were used throughout the experiments: (i) 400 μM, as in the native mass spectrometry experiments, and (ii) a concentration in which at least 95% of the protein is expected to be in complex with the effector. We monitored the limited proteolysis by taking aliquots at various time-points (2 min, 15 min, 1 hr, 6 hrs and 22 hrs), and analyzed the reaction mixture by mass spectrometry. In the C-terminal DNA-binding domain (residues 145-232), most cleavages were rapid (within 2 min and 15 min), irrespective of the absence or presence, type and concentration of phenol derivative used (Table 4.2, Fig. 4.6c). The only effect in the proximity of the DNA-binding domain that resulted from the interaction with the strong effectors 2,4-DCP, 2-Br-4-CP, 2,4,5-TCP and 2,4,6,-TCP (class III) was observed in the α-helix which connects the sensory domain with the DNA-binding domain (flexible hinge region). Here, the cleavage site R152 was fully (CHPA) or partially (other class III compounds) protected from cleavage.

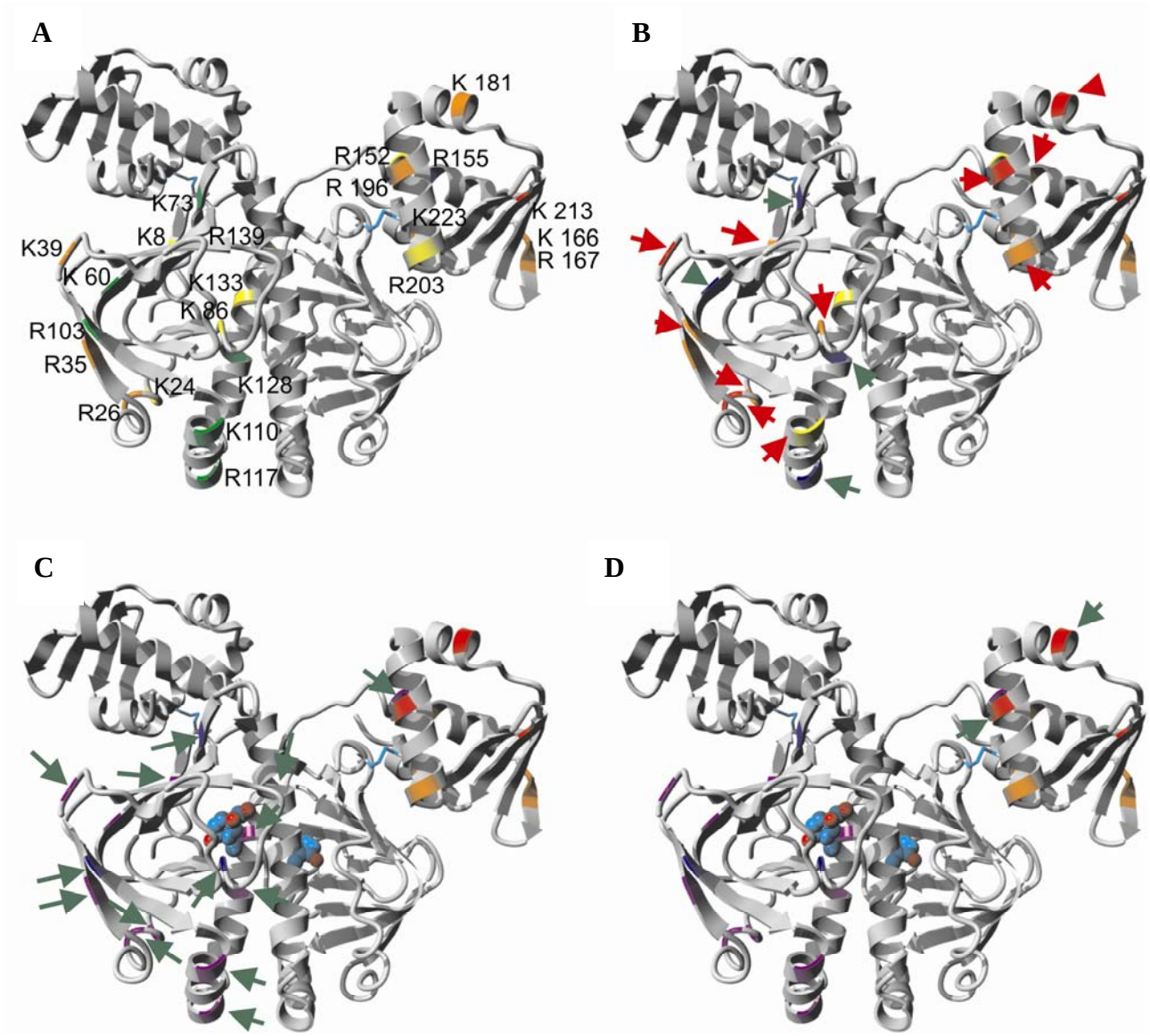


Figure 4.6 CprK1 requires a cascade of structural changes to interact with "dehalobox" DNA. The X-ray model shows CprK1 from *D. hafniense* under oxidizing conditions and in the presence of CHPA (PDB code 2H6B). The sensitivity of the different proteolytic cleavage sites are indicated in red, orange, yellow, green, blue and purple for appearance after 2 min, 15 min, 1 hr, 6 hrs, 22 hrs and no cleavage, respectively. Low abundant peptides that appeared after 2 min are indicated in dark orange. For clarity, only monomer A is colour coded. The arrows show the cleavage sites more accessible (red) or more protected (green) under the following conditions: (**A**) CprK1 under oxidizing conditions, (**B**) CprK1 under reducing conditions, (**C**) CprK1 under reducing conditions and in the presence of CHPA and (**D**) CprK1 under reducing conditions and in the presence of CHPA and 30-bp "dehalobox" DNA.

In the sensory module (residues 1-144), however, 15 effector-dependent cleavages were observed. In the presence of HPA and 3,5-DCP, compounds that do not have an allosteric effect on CprK1 (class I, Fig. 4.4), and with the moderate effector 2,3-DCP (class II), we did not observe any changes in the limited proteolysis pattern compared to the effector-free CprK1 pattern at either effector concentrations. For the strong effectors 2,4-DCP, 2-Br-4-CP, 2,4,5-TCP and 2,4,6,-TCP (class III, Fig. 4.4), several potential cleavage sites were partially protected from proteolysis in the sensory module (K39, K86, R103, K110, R117, K128, K133 and R139) in the presence of 400 µM of effector. At the concentration which is necessary to obtain at least 95% of the CprK1-DNA complex, some additional cleavage sites were partially protected (R8, R24, R26, R35, R60, R73). Incubation with the strong effector CHPA (class III) showed additional protection of most of the cleavage sites at both concentrations.

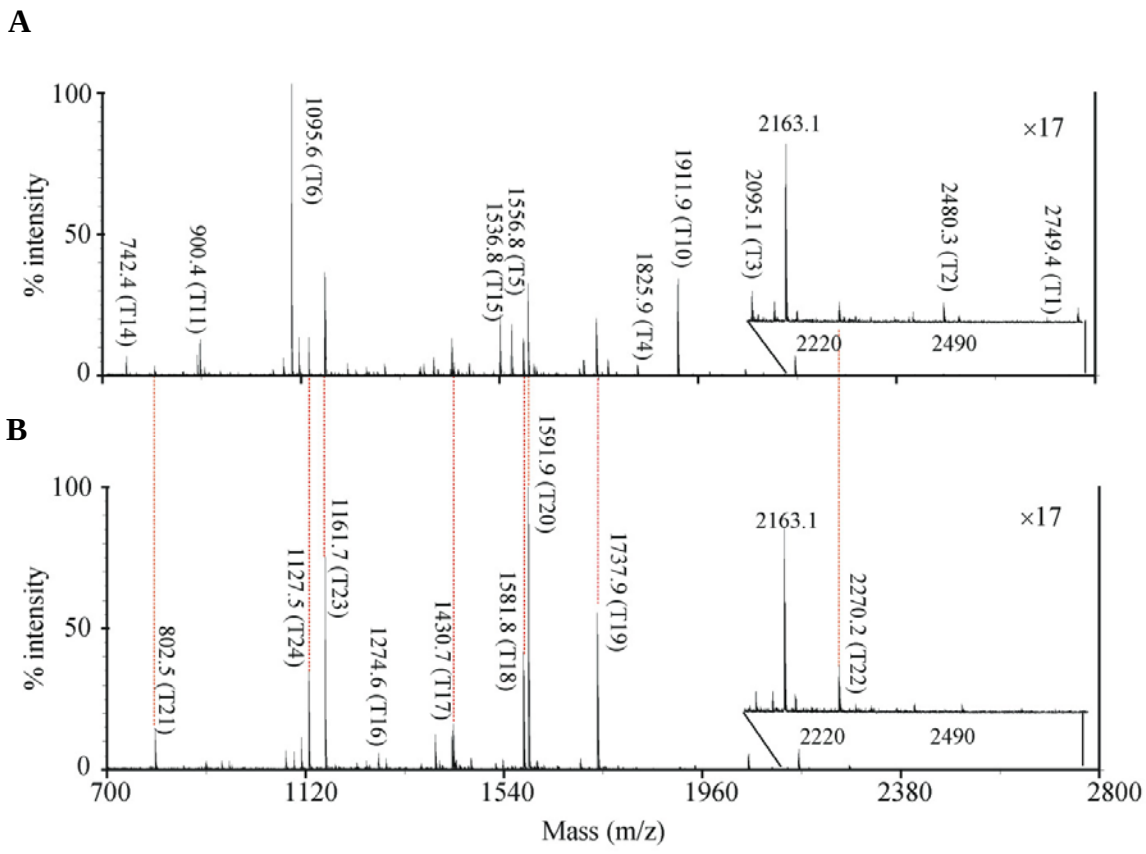


Figure 4.7 The interaction with CHPA decreases the dynamics of CprK1. Matrix assisted assisted laser desorption/ionization spectra of peptides of CprK1 after 6 hrs of limited proteolysis of native CprK1. CprK1 was incubated in a 100 mM ammonium acetate solution, pH 8 at a protein dimer concentration of 6 µM, 10 mM dithiothreitol and in the absence (**A**) or presence (**B**) of 400 µM CHPA. Trypsin was used as the proteolytic enzyme at a concentration of 0.27 µM. Peptides are labelled according to Fig. 4.5.

Figure 4.7 shows the matrix assisted laser desorption/ionization mass spectra of identified CprK1 peptides after 6 hrs of trypsin digestion of native CprK1 under reducing conditions in the absence or presence of CHPA. The spectra clearly show that only the peptides T16 to T24 in the C-terminal DNA-binding domain are sensitive for proteolytic cleavage in the presence of CHPA. In contrast, the residues in the sensory module (corresponding to peptides T1 to T15) were fully protected from cleavage in the presence of CHPA. The protected fragment T15 includes R152, a residue that is positioned in the hinge region connecting the sensory module with the DNA-binding module.

Finally, the effect of DNA binding on the quaternary structure of CprK1 was studied by limited proteolysis. When we incubated "dehalobox" DNA with CprK1 under reducing conditions, we did not observe changes within the limited proteolysis pattern compared to CprK1 in the absence of DNA, except the peptides T1 to T4, which are positioned in the N-terminal region of the sensory module were not detected in the presence of DNA (data not shown). The disappearance of the peptides corresponding to the N-terminal extremity can not be explained. Similarly, when DNA binding was studied in the presence of the most potent effector CHPA under reducing conditions, the proteolysis pattern was very similar as compared with the proteolysis pattern of the mixture of CprK1 and CHPA (Fig. 4.6d). However, peptide T20 (residues 182-196) had a markedly decreased intensity in the presence of DNA after 15 min of incubation (see Supplementary figures 4.3a and 4.5 for spectra), indicating that binding of DNA partly protected this region from trypsin digestion. T20 covers the region of the helix-turn-helix DNA-binding motif which is known to directly interact with the target DNA (recognition α helix, residues 191-204 in CprK1) according to the current model of DNA binding to CprK1.

Discussion

The transcriptional regulator CprK1 activates transcription of genes that encode proteins involved in reductive dehalogenation of chlorinated aromatic compounds (Gábor *et al.*, 2006). In spite of the X-ray models of free CprK from *D. dehalogenans* in the reduced form and the CHPA-bound CprK1 from *D. hafniense* in the oxidized form (Joyce *et al.*, 2006), the mechanism of activation, which leads to DNA binding and subsequently to transcriptional activation, is poorly understood because these two proteins are from different sources and the active form (CHPA bound CprK1 in reduced form) is not crystallized yet. However, the X-ray models suggest that effector binding to reduced CprK induces structural rearrangement in the N-terminal β-barrels, and it has been postulated that this change allows formation of interdomain contacts that disrupt the DNA-binding domain dimer interface leading to repositioning of the helix-turn-helix motif for DNA binding (Joyce *et al.*, 2006). In the present study, we characterized the CprK1-effector-"dehalobox" DNA interaction and determined the effector specificity of CprK1 by multiple mass spectrometry-based methods. We demonstrate that CprK1 requires changes in dynamics and/or conformation to interact with effector and "dehalobox" DNA, and thus to become activated.

Our macromolecular native mass spectrometry data showed that CprK1 is a dimer both under oxidizing and reducing conditions, thus the disulfide bridges that connect the two subunits of the dimer (Cys11-Cys200) are not essential for dimerization. This is in agreement with size-exclusion chromatography data from Gábor *et al.* (2006), which suggested that wild type CprK1 and the disulfide bridge-deficient mutants Cys11Ser CprK1 and Cys200Ser CprK1 exist as a homodimer. Native mass spectrometry also showed that reduction of the disulfide bridge and effector binding are simultaneously needed for efficient "dehalobox" DNA binding. Results also revealed that CprK1 interacts with one molecule of "dehalobox" DNA as a dimer.

Using a small library of potential effector molecules, we could determine the effector specificity of CprK1. Previously, two factors were presumed to determine the potential of a halogenated phenolic compound to act as an effector for CprK1: (i) the position and nature of the substitutions and (ii) the acid dissociation constant (pK_a value) of the molecule (Joyce *et al.*, 2006). On the basis of our data we concluded that two substitutions at the phenolic ring are crucial for effector activity: one at the *ortho* position (chorine or bromine) and one at the *para* position. The *ortho* chlorine atom in CHPA is positioned in van der Waals contact with the central α-helix of CprK1 which connects the effector-binding domain with the DNA-binding domain (Joyce *et al.*, 2006). The substitution at the *para* position can be a halogen or an acetic acid moiety, as was already hypothesized by Joyce *et al.* (2006). Substitutions at position 5 and/or 6 are accepted, but not required for effector activity. Our data suggest that the interaction of the effector with the protein is destabilized when a chloride group is present in position 6 in comparison to substitution in position 5, since 2,4,6-TCP showed decreased protecting effect in limited proteolysis experiments than 2,4,5-TCP. It was hypothesized that the high specificity of CprK1 towards CHPA is likely achieved by the "pK_a interrogation" mechanism (Joyce *et al.*, 2006).

The specific recognition of CHPA would be based on the ability of CHPA to ionize to the phenolate form via deprotonation at the hydroxyl group (pK_a 8.3) and to consequently interact with K133. Our study confirmed that HPA, the dechlorinated derivative of CHPA with pK_a 10.3, is not an effector for CprK1, since under physiological pH it mostly exists in its protonated form which does not enable the molecule to make the essential salt bridge with K133. Likewise, all the phenol derivatives that proved to be a strong effector for CprK1 have pK_a values lower than 8.3. There was one exception: 3,5-DCP was not an effector although its pK_a value of 8.3 would allow the molecule to be present in the phenolate form. Our library of potential effectors shows that both the ability to form a phenolate and the presence of the chloride group in *ortho* position are equally important factors that allow DNA binding.

Next, we used limited proteolysis with mass spectrometry to monitor structural dynamics and conformational changes of CprK1 in a medium-throughput fashion (Hubbard, 1998). Three consecutive steps were examined: the effect of reduction on CprK1 structure, the result of effector binding, and finally the effect of DNA binding to the protein. The results allowed us to propose a working model that represents the mechanism of activation and dynamics of CprK1 (Fig. 4.8).

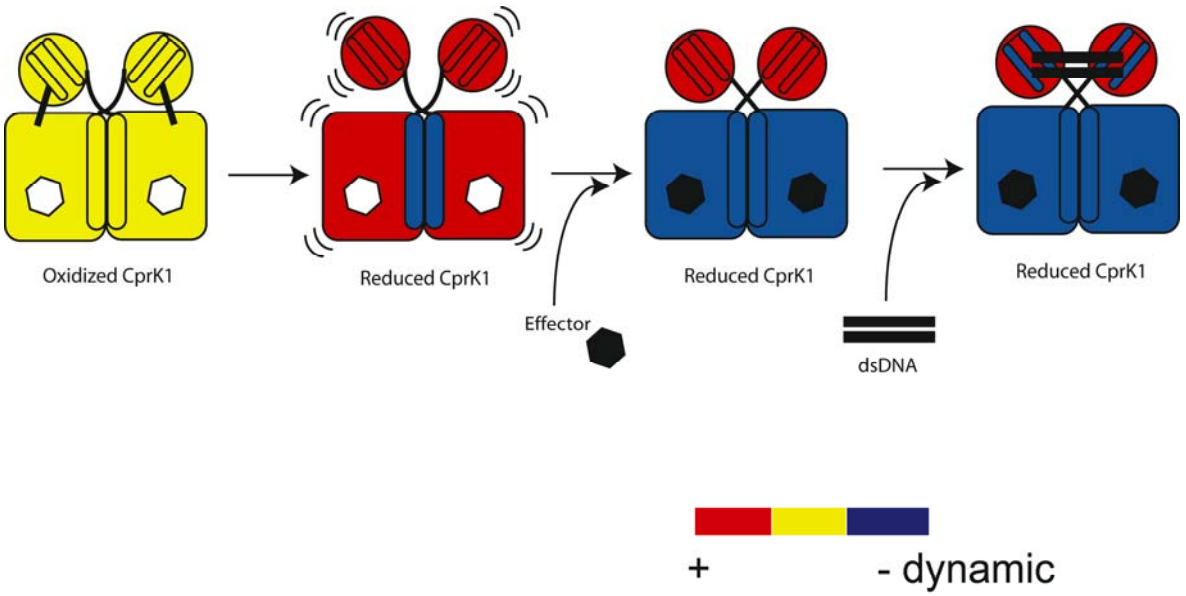


Figure 4.8 Model describing the mechanism of activation of CprK1. The model presents the dynamics and/or conformational changes in CprK1 that are required for "dehalobox" DNA binding. The reduction of oxidized enzyme, containing a disulfide bridge between residues Cys11 and Cys200, results in a conformational rearrangement and increased flexibility in most regions of the protein. The α-helix forming the dimeric interface, however, shows an increased stability. The binding of an effector molecule to CprK1 induces protection and local changes in the N-terminal effector-binding domain that are translated to the C-terminal DNA-binding domain by changes in inter-domain or interface interactions and lead to the required conformational changes for DNA binding in the DNA-binding domain and thus transcriptional activation. Finally, the binding of the "dehalobox DNA" shows only the protection of the peptide included in the helix-turn-helix motif. The dynamics of CprK1 dimer is indicated in blue, yellow and red for low, medium and high flexibility.

Oxidized CprK1 has an overall rigid structure due to the presence of the disulfide bridge Cys11-Cys200 connecting the N-terminal extremity of a monomer with the DNA-binding helix-turn-helix motif of the other monomer. The few areas accessible to digestion in the oxidized protein were located at the surface of the protein (Fig. 4.7a). Protected areas included the dimerization α-helix and the β-barrels in the effector-binding domain. Notable was the strong protection of the R155 residue in the C-terminal DNA-binding domain of the protein.

Under reducing conditions, the disulfide bonds are disrupted and the protein seems to become overall more flexible. The cleavage frequency increased in most regions of both the N-terminal effector-binding and C-terminal DNA-binding domains, including the R155 residue. The X-ray structure of the reduced protein from *D. dehalogenans* reveals interactions between the DNA-binding domains of the two CprK1 monomers (residues from helices 152-164 and 180-187 buried) (Joyce *et al.*, 2006) but from limited proteolysis results these interactions seem to be not stable (lack of protection at residues R155, R167, K181 under reducing conditions). Intriguiungly, the dimerization α-helix became more protected when the disulfide bonds were reduced, indicating that packing interactions in the monomer-monomer interface became tighter upon reduction. Joyce *et al.* (2006) showed that the positions of the dimerization α-helices were the same in both the reduced and the oxidized structure, thus their observed tighter interaction probably allows the protein to be more flexible in other regions without loosing its stability. In addition, the region around K60 and K73 in the effector-binding domain became less sensitive for proteolytic cleavage upon reduction. The reason for this remains elusive.

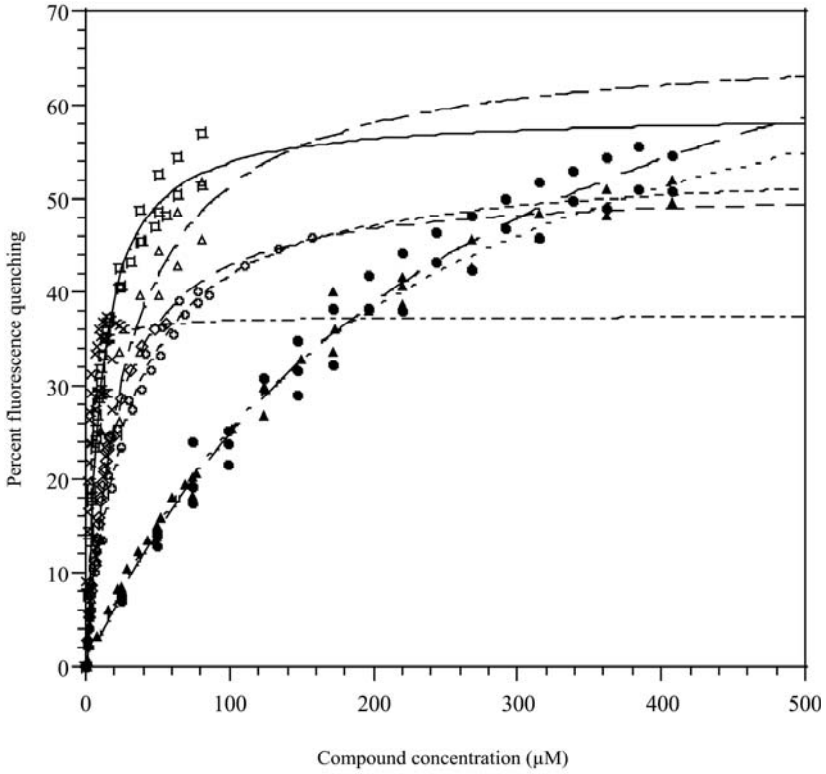
Although the reduction of the protein is a prerequisite for DNA binding, our results indicate that it has to be accompanied by the binding of a suitable effector molecule to enable CprK1 to form complex with its target DNA. Effector binding induced structural and/or conformational changes in the sensory module of CprK1, shown by the protection of potential cleavage sites in this domain upon effector binding. Protection was the most effective with the strong effector molecules 2,4-DCP, 2-Br-4-CP, 2,4,5-TCP and 2,4,6,-TCP (class III, determined by native mass spectrometry). Thus, the effector-binding domain shifts to a more rigid or compact structure. This is in agreement with the X-ray structures, which showed that the binding of CHPA reduces the volume of the hydrophobic pocket (shift in the position of G85, reorientation of Y130 and L131) and stabilizes the structure by more interaction (K86 interacts with the acetic group, which is also hydrogen bound to T90 and N92). Moreover, we observed a strong stabilization of the interface α-helix. In contrast, the C-terminal DNA-binding domain proved to be sensitive to protease digestion, irrespective of the absence or presence of effectors. However, an important exception was R152, a residue situated in the hinge region which connects the sensory domain with the DNA-binding domain in CprK1. This arginine was fully protected from cleavage in the presence of CHPA, although it is not in direct interaction with the effector itself. This suggests that this region might be involved in transmitting the effector binding signal from the sensory domain to the DNA-binding domain.

Indeed, mutations in the corresponding hinge region of *E. coli* CRP (A144T) and *Listeria monocytogenes* PrfA (G145S) resulted in a constitutively active DNA-binding conformation of these proteins, giving further support to our hypothesis (Eiting *et al.*, 2005; Weber *et al.*, 1987).

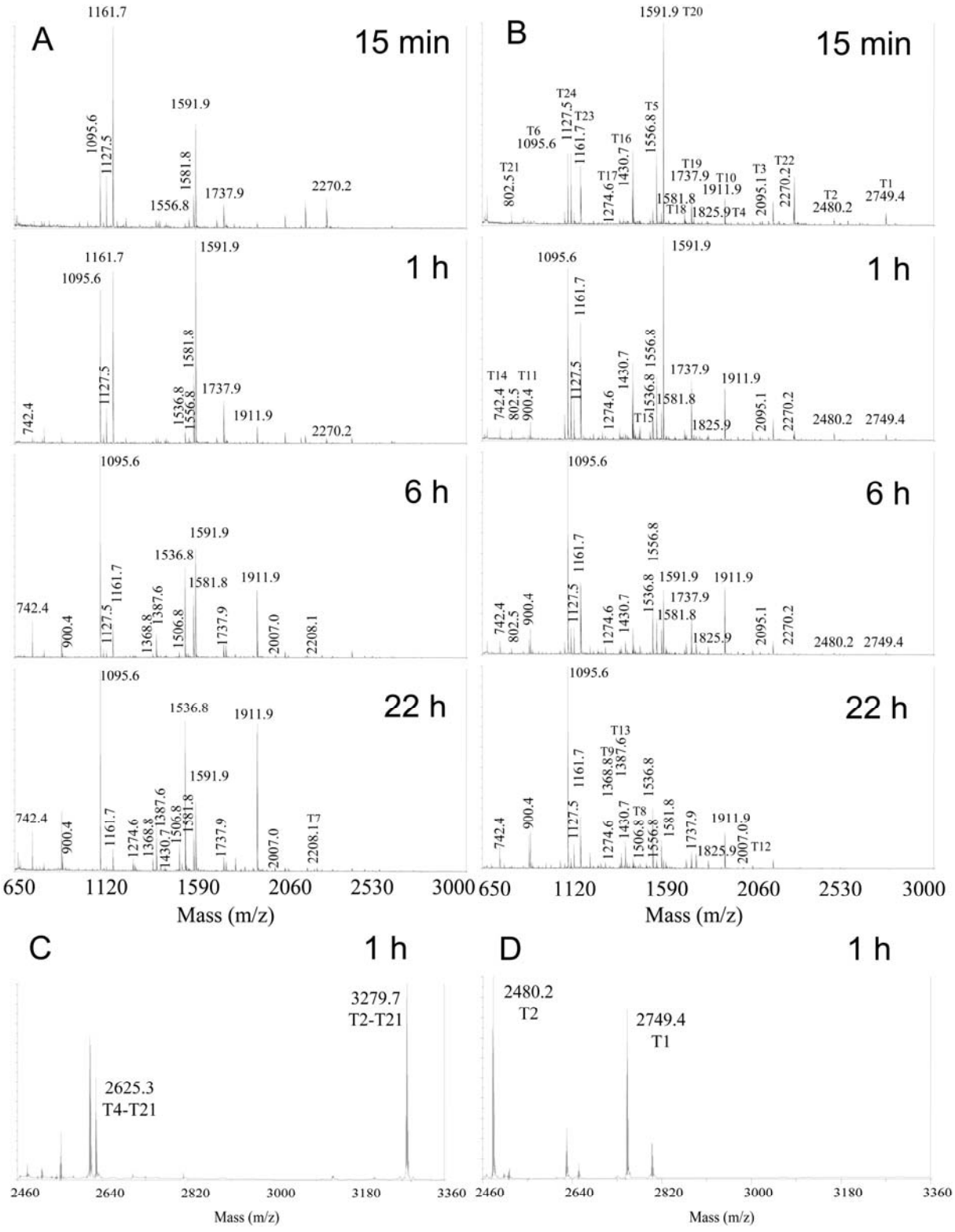
Finally, we showed that DNA binding protected a 14-amino acid peptide (T20) from trypsin digestion. This peptide is part of the recognition α helix in the helix-turn-helix DNA-binding motif of CprK1 and contains the V--SR conserved motif which is known to be involved in contacting the specific TTAAT----ATTAA "dehalobox" DNA sequence (Gábor *et al.*, 2006). The results of the limited proteolysis experiments give evidence that CprK1 makes a direct contact with its specific DNA target via the second helix of the HTH motif, similarly to *E. coli* CRP and FNR proteins (Green *et al.*, 2001).

In conclusion, the presented multiplexed mass spectrometry approach and the small library of effectors used allowed comprehensive analysis of ligand- and redox-controlled induction of active CprK1 conformations. Limited proteolysis provides more details about the dynamics involved in CprK1 activation. This extends our knowledge not only on the molecular basis of transcriptional activation of halorespiration by CprK1, but also generally on the structure-function relationship of its related members from the CRP-FNR family.

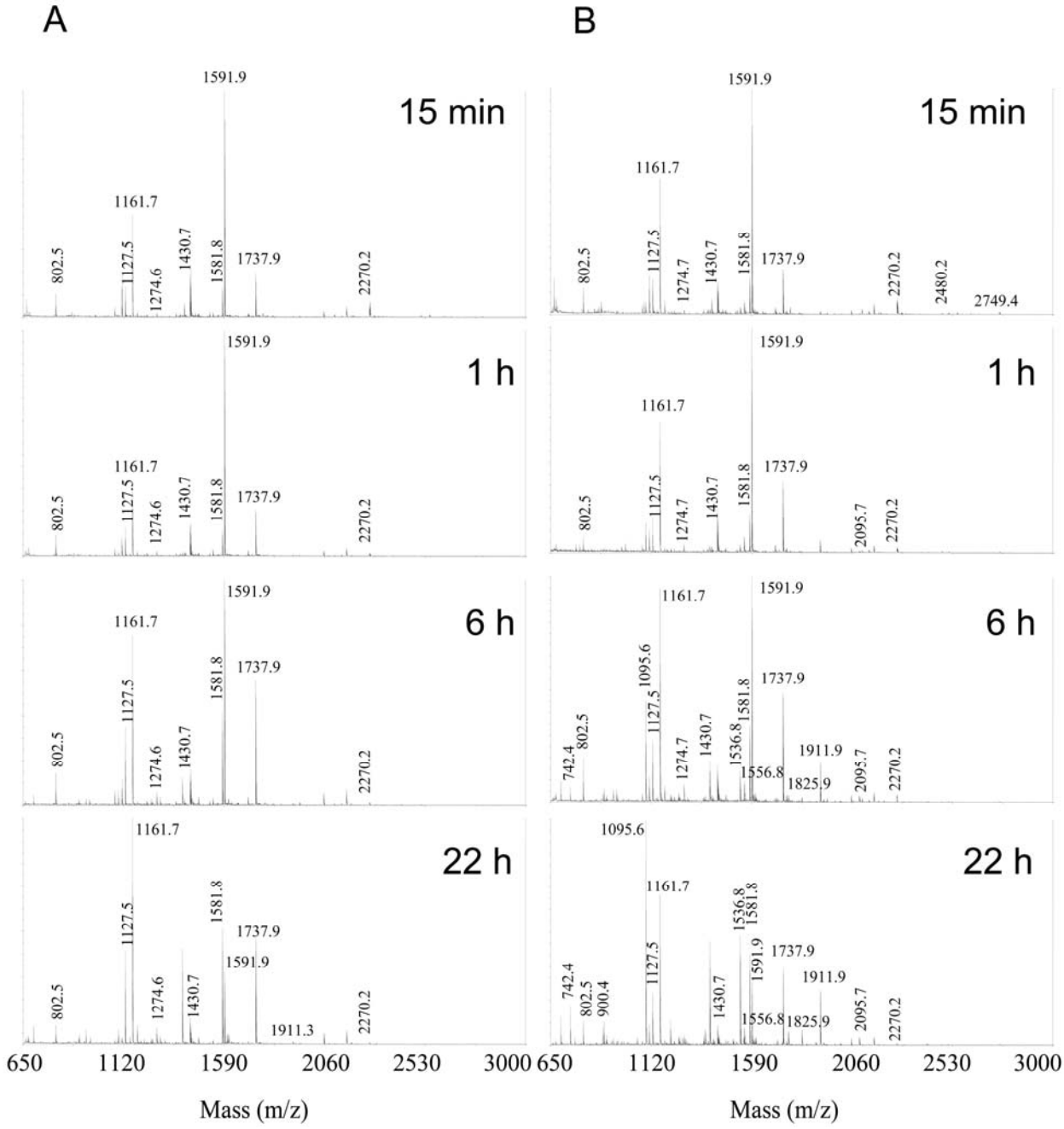
Appendix



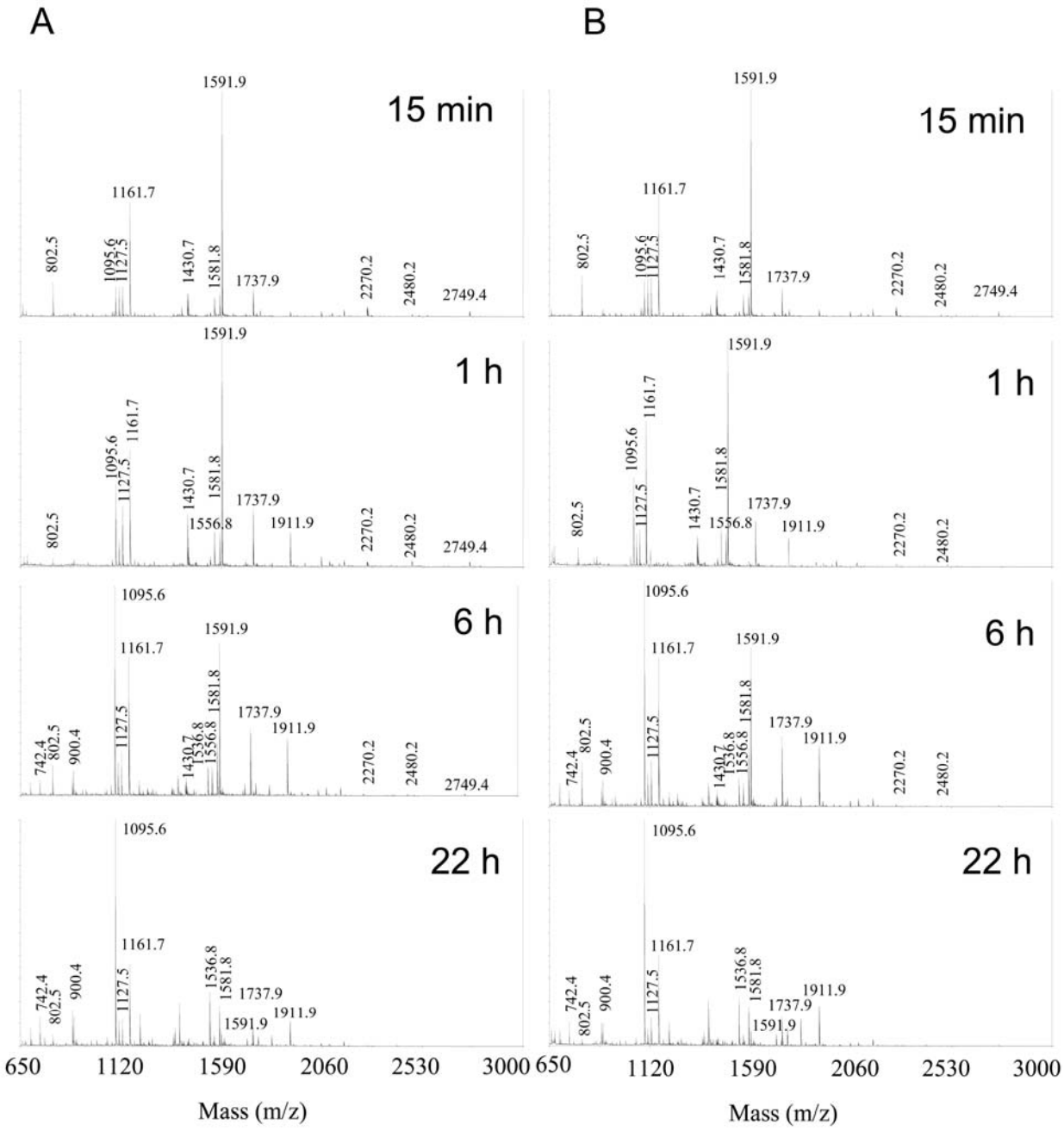
Supplementary figure 4.1 Potential effectors titration curves of reduced CprK1 based on quenching of fluorescence emission monitored at 330 nm. The protein (0.2 μM final concentration) is incubated in the 100 mM ammonium acetate buffer pH 8.0 in the presence of 0.75 mM DTT. The data points are as follows: open square, 2,4,5-TCP; open triangle, 2,4,6-TCP; open diamond, 2,4-DCP; open circle, 2-Br-4-CP; filled triangle, 2,3-DCP; filled circle, 3,5-DCP; cross, CHPA.



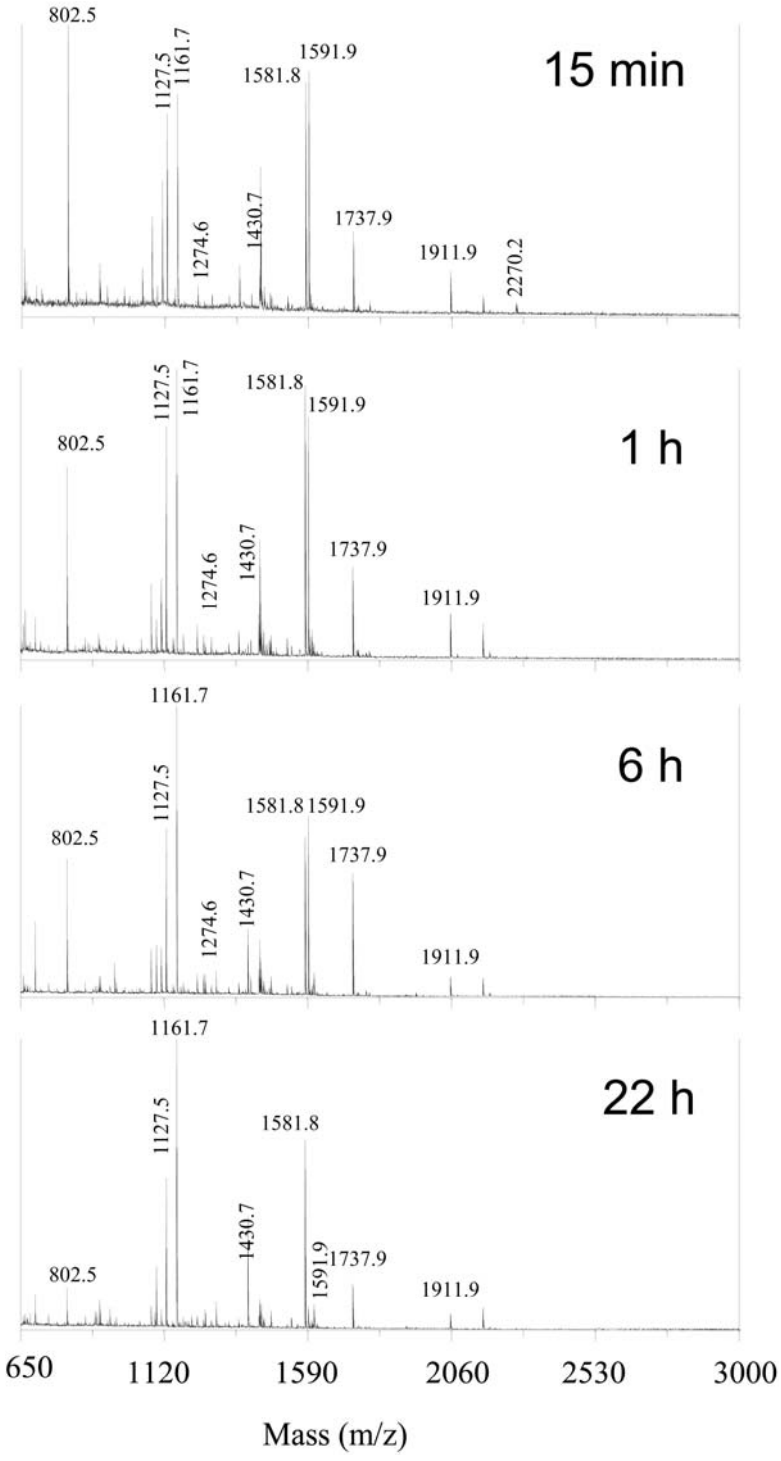
Supplementary figure 4.2 Range 650-3000 m/z MALDI mass spectra of the Trypsin reaction mixture after time 15 min, 1 h, 6 h and 22 h of CprK1 in 100 mM ammonium acetate pH 8 in the absence (**A**) or the presence (**B**) of 10 mM DTT. Range 2460-3360 m/z MALDI mass spectra of the Trypsin reaction mixture after time 1 h of CprK1 in the absence (**C**) or the presence (**D**) of 10 mM DTT.



Supplementary figure 4.3 Range 650-3000 m/z MALDI mass spectra of the Trypsin reaction mixture after time 15 min, 1 h, 6 h and 22 h. CprK1 in 100 mM ammonium acetate pH 8, 10 mM DTT, in the presence of CHPA (**A**) or the presence of 2,4,5-TCP (**B**).



Supplementary figure 4.4 Range 650-3000 m/z MALDI mass spectra of the Trypsin reaction mixture after time 15 min, 1 h, 6 h and 22 h. CprK1 in 100 mM ammonium acetate pH 8, 10 mM DTT, in the presence of 2,4-DCP (**A**) or the presence of 2,4,6-TCP (**B**).



Supplementary figure 4.5 Range 650-3000 m/z MALDI mass spectra of the Trypsin reaction mixture after time 15 min, 1 h, 6 h and 22 h. CprK1 in 100 mM ammonium acetate pH 8, 10 mM DTT, in the presence of CHPA and "dehalobox" DNA.

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Divergence of multiple CprK-paralogues in *Desulfitobacterium hafniense*

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Abstract

Gene duplication and horizontal gene transfer play an important role in the evolution of prokaryotic genomes. We have investigated the role of three CprK-paralogues from the CRP-FNR family of transcriptional regulators that are encoded in *Desulfitobacterium hafniense* DCB-2 and possibly regulate expression of genes involved in the energy-conserving terminal reduction of organohalides (halorespiration). The results show that two regulators (CprK1 and CprK2) have an overlapping effector-specificity with preference to an *ortho*-chlorophenol, while *meta*-chlorophenols proved to be effectors for CprK4. The presence of two potential transposase-encoding genes in the vicinity of the *cprK* genes indicates that their redundancy is probably caused by mobile genetic elements. The CprK-paralogues activated transcription from promoters containing a 14-bp inverted repeat (dehalobox) that closely resembles the FNR-box. We found a strong negative correlation between the rate of transcriptional activation and the number of nucleotide changes from the optimal dehalobox sequence (TTAAT-N_4-ATTAA). A strong halorespiration-inducible promoter was found upstream of a gene that encodes a methyl-accepting chemotaxis protein, which is the first indication for taxis of an anaerobic bacterium to halogenated aromatic compounds.

Introduction

Members of the CRP-FNR family of transcriptional regulators show exceptional diversity in their domain structure, effector specificity and target promoters. As such, they control a wide range of physiological processes (Korner *et al.*, 2003). FNR of *Escherichia coli* is known to regulate the transcription of more than 120 genes that are involved in anaerobic respiration (Bauer *et al.*, 1999). FNR senses redox changes via a prosthetic group, i.e. an iron-sulphur center that is bound by a conserved cluster of cysteine residues located near its N-terminal end (Kiley *et al.*, 1998). CRP of *E. coli* is activated directly by the allosteric conformational change induced by cAMP binding, which enables CRP to respond to glucose starvation (Kolb *et al.*, 1993). At present, over 350 members of the CRP-FNR family have been identified, with many examples of more than one CRP-FNR homologue in the same organism (Korner *et al.*, 2003). The relationship between transcriptional regulators that belong to the same family and are encoded in the same organism is often complex, showing hierarchical characteristics. The facultative anaerobic γ-proteobacterium *Pseudomonas stutzeri* encodes four FNR homologues: FnrA, which contains the N-terminal cysteine motif for iron-sulphur binding and three DNR homologues that lack the cysteine-based sensory module (Vollack *et al.*, 1999). DnrS is involved in oxidative stress response, and the expression of its gene is activated by FnrA. DnrE is activated in nitrate-challenged cells under aerobic conditions, and is partly under the control of the nitrate response regulator NarL. The third regulator, DnrD, plays a key role in controlling denitrification and does not appear to be hierarchically controlled by other proteins.

Another example for a regulatory cascade mediated by CRP-FNR homologues is found in *Rhodopseudomonas palustris* (Egland *et al.*, 2000). In this facultative anaerobe α-proteobacterium, the degradation of 4-hydroxybenzoate is regulated by the CRP-FNR-type HbaR, which is hierarchically controlled by the oxygen-sensing AadR protein, also from the same family.

The growing number of sequenced bacterial genomes expands the CRP-FNR family even further (Korner *et al.*, 2003). Genome analysis of *E. coli* has identified the third member of the CRP-FNR family: YeiL (Anjum *et al.*, 2000). Studies of transcriptional fusions (*yeiL::lacZ*) in cells with different genotypes suggest that YeiL is a nitrogen starvation regulator, which is positively regulated by the stationary-phase sigma factor RpoS as well as by Lrp and IHF. Besides *Bradyrhizobium japonicum* and *Magnetospirillum magnetotacticum*, the chromosome of *Desulfitobacterium hafniense* DCB-2 shows the highest diversity of CRP-FNR-type regulators encoded in a single host (Mesa *et al.*, 2006). *D. hafniense* belongs to the lineage of gram-positive low G+C bacteria, and its most important characteristic is the capability of anaerobic respiration with halogenated compounds as terminal electron acceptors (halorespiration) (Christiansen *et al.*, 1996). This strictly anaerobic bacterium can couple the reductive dehalogenation of often toxic *meta*- and *ortho*-substituted phenol derivatives to its growth, providing a potential means of bioremediation of polluted anoxic environmental sites (Van Eekert *et al.*, 2001). The key enzymes in halorespiration are the reductive dehalogenases (RD), corrinoid/iron-sulphur containing proteins that are predicted to be membrane anchored by a small protein, the gene of which generally clusters with the RD-encoding gene (Smidt & de Vos, 2004). From *D. hafniense* DCB-2 a halorespiration inducible *ortho*-chlorophenol reductive dehalogenase (CprA1) has been isolated and characterised (Christiansen *et al.*, 1998). The transcription of *cprA1* is activated by CprK1, a new member of the CRP-FNR family, in the presence of 3-chloro-4-hydroxyphenylacetic acid (CHPA) (Gábor *et al.*, 2006). It was shown that binding of CHPA to CprK1 results in an active DNA-binding conformation which enables the regulator to activate transcription from promoters that contain a specific DNA sequence termed the dehalobox (TTAAT-N_4-ATTAA) (Gábor *et al.*, 2006). The partially available sequence data of the genome of *D. hafniense* DCB-2 revealed the presence of at least 20 proteins that fulfil selection criteria for the CRP-FNR family, namely (i) a length of 230-250 amino acids, (ii) a carboxyl-terminal helix-turn-helix DNA-binding motif, and (iii) an amino-terminal nucleotide (effector)-binding domain (Korner *et al.*, 2003). Five of the CRP-FNR family members (including CprK1) show high homology with CprK from *D. dehalogenans* (Pop *et al.*, 2004) and with one exception, they cluster with genes that code for potential halorespiration proteins (Villemur *et al.*, 2002). This unusually high occurrence of CprK-like regulators is likely to be correlated with the relatively high number of halogenated compounds that this organism can respire, enabling specific response by each regulator to a specific group of halogenated compounds (Madsen *et al.*, 1992).

The aim of the present paper was to investigate the role of multiple CprK-paralogues in *D. hafniense* by using *in vivo* promoter-probe assays as well as *in vitro* DNA-binding assays. Our results indicate that at least two of the four CprK-paralogues have a distinct effector range, which would reflect the gain of new or specific function by divergence of redundant genes.

Materials and Methods

Bioinformatics and statistical tools

The genome sequence of *Desulfitobacterium hafniense* DCB-2 was screened for halorespiration genes using basic local alignment search tool (BLAST) (McGinnis *et al.*, 2004). Protein accession numbers of the identified CprK homologues (based on *D. hafniense* genome sequence version 24-Jun-2005): ZP_00558871.1 (CprK1), ZP_00558892.1 (CprK2), ZP_00558887.1 (CprK3), ZP_00560926.1 (CprK4), and ZP_00559167.1 (CprK5). Conserved protein domains were identified with the help of the Pfam database (http://www.sanger.ac.uk/Software/Pfam), and HNN secondary structure prediction tool (http://npsa-pbil.ibcp.fr). Multiple sequence alignment was produced by ClustalX 1.81 (Thompson *et al.*, 1997) and edited in GeneDoc 2.6 (Nicholas *et al.*, 1997). Correlation coefficients were computed by using Excel (Microsoft Office 2003).

Identification of putative regulator binding DNA sequences

The training datasets for the motif discovery MEME algorithm (Bailey *et al.*, 1994) consisted of 40 DNA sequences with an average size of 150 bp from direct upstream region of the translational start codon of halorespiration genes. As a positive control, sequences that contained known FNR-like regulatory boxes from *D. dehalogenans* chlorophenol reductive dehalogenase (*cpr*) gene cluster were included in the analysis (Smidt *et al.*, 2000). Additionally, manual detection of inverted repeats was performed by Palindrome tool (http://bioweb.pasteur.fr/seqanal/interfaces/palindrome.html).

Overproduction and purification of CprK-like proteins

CprK1 was prepared as described previously (Gábor *et al.*, 2006). The genes coding for CprK2-CprK5 were amplified from *D. hafniense* genomic DNA using oligonucleotide primers as listed in Table 5.1. After digestion with the appropriate endonucleases, PCR products were cloned into linearized pET24d expression vectors (Novagen). The resulting T7-based expression vectors containing one of the *cprK* homologues (Table 5.2) were introduced into *E. coli* JM109(DE3) cells by heat-shock transformation. Overproduction and purification of the recombinant proteins were done essentially as described for CprK1 (Gábor *et al.*, 2006), with the exception that IPTG-induced CprK2 and CprK4 production was carried out at 37 °C instead of 20 °C. Efforts to produce CprK3 and CpK5 as a soluble protein were not successful. Purification of CprK2 and CprK4 was performed similarly to CprK1, using sequential HiPrep heparin and Superdex 200 (Amersham Biosciences) chromatography columns (Gábor *et al.*, 2006).

Table 5.1 Oligonucelotide primers used in the study.

Primers	Sequence[a]	Orientation[b]
BG1376	CGCGCCCATGGATGCTAATAGGAGTTATTGTATTG	S
BG1377	CGCGCGAATTCGAGCAATTTATACTATGATTAATGAG	A
BG1385	CGCGCCCATGGAAAGGGTAATAAGCAATCAC	S
BG1388	GCGCGCCATGGATGACGGTGTAAAGAGAATCATTC	S
BG1389	GCGCGGGATCCGAATATTCGATAATCTCATTGATCAAGC	A
BG1391	GCGCGCCATGGGAGAAATTCTTAAAAATTATATTTTTCC	S
BG1748	GGTTGAGAAATTCAGGTAAAG	S
BG1749	GGATCACATACGCAAGTATTAATG	A
BG1826	CGCGCAAGCTTGCTATGATGTTTATCTTCCTCC	S
BG1827	CGCGCGGATCCGGACGGCATCCTTCCTTTG	A
BG1828	GCGCGAAGCTTGGCCGGTCTTGTTGCCC	S
BG1830	CGCGCGGATCCGCTTCACCAGGAAAGGCGG	A
BG1831	GCGCGAAGCTTGCTCTTCTCGAAGAGGGGATAGC	S
BG1832	CGCGCGGATCCCAGGATAGTTCCCATTTTTTCACC	A
BG1835	GCGCGAAGCTTCCGAGGTTGAGAGCTTTAATTG	S
BG1836	CGCGCGGATCCGTACTCATCCCTTTCACCTCC	A
BG1838	GCGCGAAGCTTGGCCCCTAATTTATGGAG	S
BG1839	CGCGCGGATCCGCTAAAAACACAATCCCGGAC	A
BG1840	GCGCGAAGCTTCCTGAAGAGAGCCCTTGATC	S
BG1841	CGCGCGGATCCGGAATGAGCGGATCTTGAATG	A
BG1842	GCGCGAAGCTTGCAACCAGCGGCTTCGCC	S
BG1843	CGCGCGGATCCGATATGGATTGCACTAAGTTCCC	A
BG1940	GCGCAAGCTTAATAAGCTATCCCCTCTTCG	A
BG1941	GCGCGGATCCTCAGAATCTCAATTCCTCTTCC	A
BG2109	GCTCATTTTCCAAATTGGCG	S
BG2110	GGTACCAGAATAGTATAAAG	A

[a] Introduced endonuclease restriction sites are underlined. [b] S, sense primer; A, antisense primer.

Table 5.2 Plasmids used in this study

Plasmids	Description	Primers	Reference
pET24d	Expression vector (5.3 kb), pMB1 ori, Kan^R, IPTG-inducible T7 promoter	-	Novagen
pAK80	Promoter probe shuttle vector (11.0 kb), p15A/*L. lactis* ori, Ery^R, promoterless l*acLM* genes	-	(Israelsen *et al.*, 1995)
pWUR90	*cprK3* gene cloned in pET24d	BG1388/1389	this study
pWUR91	*cprK5* gene cloned in pET24d	BG1376/1377	this study
pWUR166	DB7:*lacLM* promoter fusion containing pAK80 derivative	BG1704/1743	(Gábor *et al.*, 2006)
pWUR168	DB6:*lacLM* promoter fusion containing pAK80 derivative	BG1702/1742	(Gábor *et al.*, 2006)
pWUR171	DB5:*lacLM* promoter fusion containing pAK80 derivative	BG1699/1782	(Gábor *et al.*, 2006)
pWUR176	*cprK1* gene cloned in pET24d	BG1379/1814	(Gábor *et al.*, 2006)
pWUR216	DB8:*lacLM* promoter fusion containing pAK80 derivative	BG1826/1827	this study
pWUR218	DB9:*lacLM* promoter fusion containing pAK80 derivative	BG1828/1830	this study
pWUR219	DB10:*lacLM* promoter fusion containing pAK80 derivative	BG1831/1832	this study
pWUR220	DB1:*lacLM* promoter fusion containing pAK80 derivative	BG1835/1836	this study
pWUR221	DB2:*lacLM* promoter fusion containing pAK80 derivative	BG1838/1839	this study
pWUR222	DB3:*lacLM* promoter fusion containing pAK80 derivative	BG1840/1841	this study
pWUR223	DB4:*lacLM* promoter fusion containing pAK80 derivative	BG1842/1843	this study
pWUR226	*cprK4* gene cloned in pET24d	BG1391/1940	this study
pWUR227	*cprK2* gene cloned in pET24d	BG1385/1941	this study

Electrophoretic mobility shift assay (EMSA)

The 85-bp dsDNA fragment containing DB8 dehalobox was PCR-amplified from *D. hafniense* genomic DNA using oligonucleotide primers BG2109 and BG2110. The 52-bp PCR product containing DB7 was obtained using BG1748 and BG1749 primers. DNA fragments were purified according to the modified "crush and soak" method and 5'-labelled using [γ-^{32}P] ATP as described previously (Gábor *et al.*, 2006). EMSA reaction mixtures (20 µl) contained 1 x POP buffer (20% glycerol, 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2.5 mM EDTA, 250 mM NaCl), 2.5 mM DTT, 1 µg poly(dG-dC)-poly(dG-dC), 0.5 µM purified CprK1 or 1 µM purified CprK4 and 1.5 to 2 nM ^{32}P-labelled DNA. Additionally, potential effector molecules were added from a 2.7 mM aqueous stock solution, to a final effector to protein molar ration of 800:1. The reaction mixtures were first incubated at 24°C for 30 min to allow complex formation, then loaded on a 6% polyacrylamide gel buffered with 89 mM Tris and 89 mM boric acid (app. pH 8.1) and electrophoresed at 10 mA constant current at 4°C, followed by drying and autoradiography.

In vivo promoter probe assays

DNA fragments carrying potential promoter elements and CprK-binding sites (dehaloboxes) were PCR-amplified from *D. hafniense* genomic DNA, digested by endonucleases followed by ligation with linearized pAK80 promoter probe vectors. The oligonucleotide primers and the resulting promoter probe vectors are listed in Table 5.1 and Table 5.2, respectively. *E. coli* JM109(DE3) cells were co-transformed with the pAK80-derivatives and the corresponding expression vectors carrying a *cprK*-homologue as indicated: pWUR226 with either pWUR216, pWUR218 or pWUR219; and pWUR227 with pWUR220, pWUR221, pWUR222 or pWUR223. Selection pressure during aerobic growth in Luria Bertani medium was maintained by the addition of 30 µg/ml kanamycin (pET24d derivatives) and 200 µg/ml erythromycin (pAK80-derivatives). Triplicate experiments were carried out for each condition, and β-galactosidase activity was measured as described previously (Gábor *et al.*, 2006). Throughout the experiments, 1 Miller unit was defined as follows: $1000 \times A_{420} / (t \,.\, v \,.\, OD_{600})$ where A_{420} is absorbance at 420 nm, t is reaction time, v is sample volume and OD_{600} is the optical density of the culture at the time the sample was taken. In control experiments, recombinant cells carrying pET24d vector instead of pWUR226 or pWUR227 were used.

Results

CprK-like regulators and target sequences in D. hafniense

The partially sequenced genome of the halorespiring *D. hafniense* DCB-2 contains five ORFs (*cprA1-cprA5*) that share 36 to 91% amino acid sequence identity with the *ortho*-chlorophenol reductive dehalogenase CprA from *D. dehalogenans*. Blast search also identified several clustering genes that, similarly to the previously annotated *cprTKZEBACD* cluster of *D. dehalogenans* (Smidt *et al.*, 2000), were predicted to encode: small hydrophobic membrane anchors for the reductive dehalogenase (*cprB*), GroEL-type chaperons (*cprD* and *cprE*), proline *cis/trans* isomerases (*cprT*), and putative transcriptional regulators from the NosR/NirI family (*cprC*) and the ubiquitous CRP-FNR superfamily (*cprK*) (Fig. 5.1a).

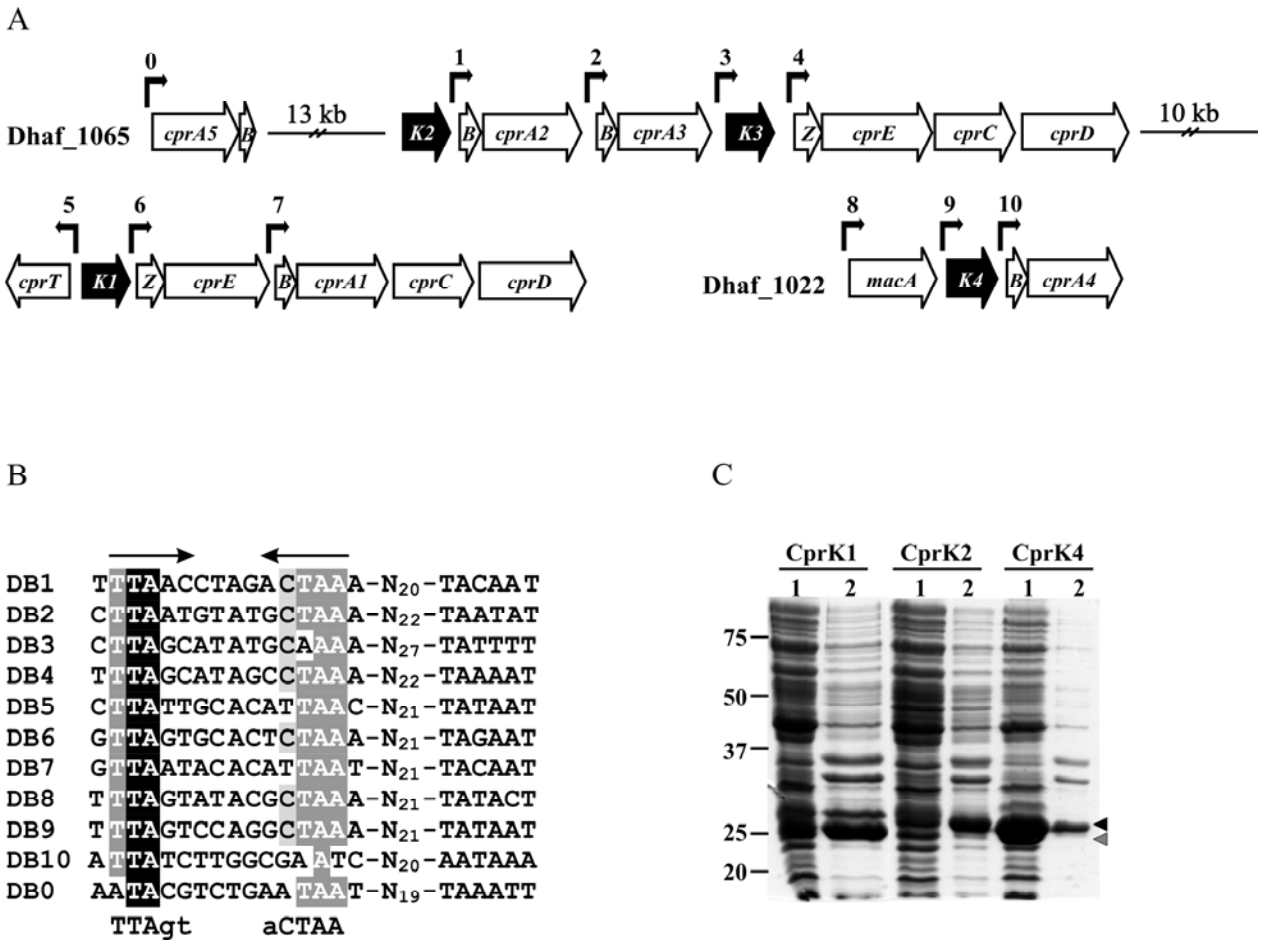

Figure 5.1 Identification and production of CprK homologues. (**A**) Reductive dehalogenase gene clusters in *Desulfitobacterium hafniense* DCB-2. Arrows indicate putative CprK-binding sites (dehaloboxes). (**B**) Sequence alignment of dehaloboxes. Arrows are placed above the 5-bp inverted repeats, the 3' nucleotides correspond to putative Pribnow boxes. Consensus sequence for dehaloboxes is shown below the alignment. (**C**) SDS-PAGE gel of the soluble (1) and insoluble (2) cell fraction of recombinant *E. coli* JM109(DE3) cells overproducing CprK1, CprK2 or CprK4. Full size heterologously-produced proteins are indicated by solid arrowhead, shaded arrowhead corresponds to the truncated CprK1 protein.

All the five *D. hafniense* CprK-paralogues (CprK1-K5) that show 36-89% amino acid sequence identity with the transcriptional regulator CprK from *D. dehalogenans* were predicted to contain an N-terminal effector binding domain (Pfam number PF00027; cNMP-binding domain) and a conserved C-terminal helix-turn-helix (HTH) DNA-binding motif (Pfam number PF00325). Indeed, the recently solved crystal structure of CprK1 from *D. hafniense* confirmed the presence of these domains (Joyce *et al.*, 2006).

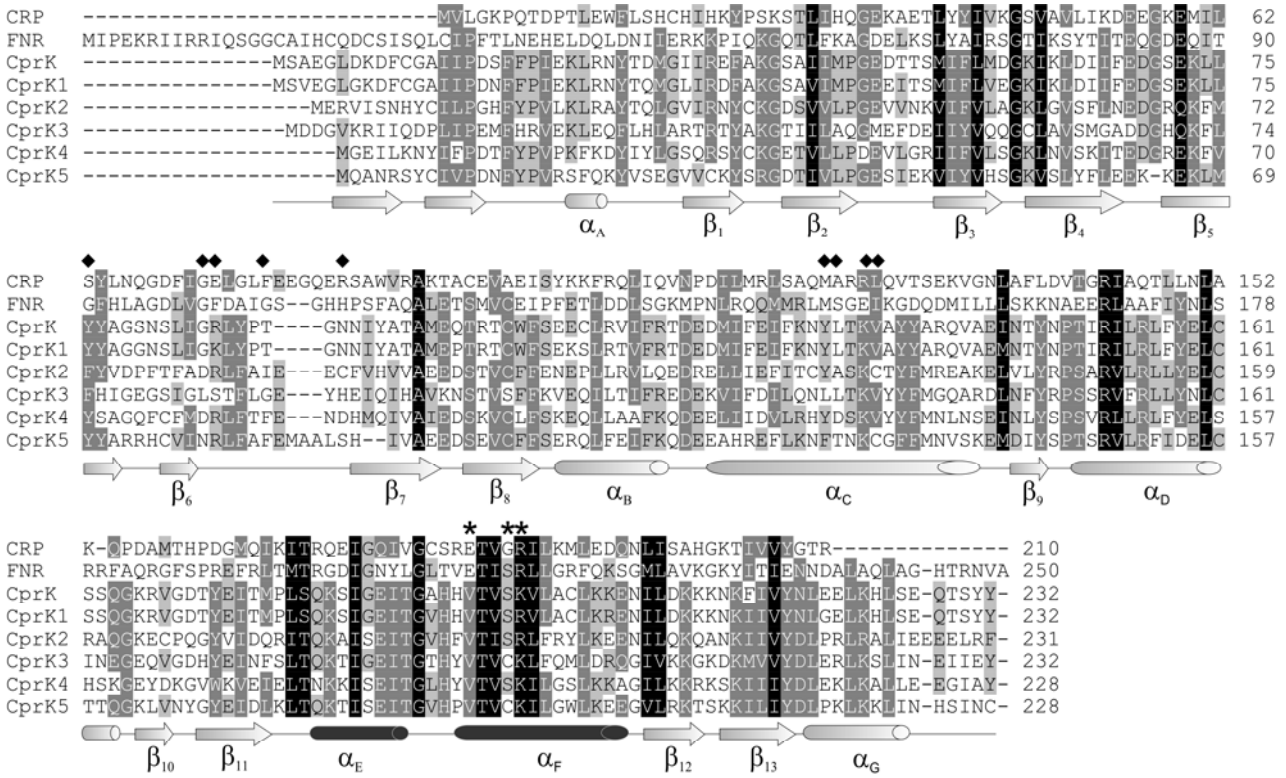

Figure 5.2 Multiple alignment of the protein sequence of selected members from the CRP-FNR family. The secondary structure of CprK1 is included below the alignment (based on PDB 2H6B, monomer A), α-helixes are denoted as cylinders, β-sheets as arrows. The cylinders corresponding to the characteristic helix-turn-helix DNA-binding motif are in black. Residues that are involved in 3-chloro-4-hydroxyphenylacetic acid binding in CprK1 are indicated with diamonds, while asterisks highlight amino acids that confer DNA-binding specificity to FNR and to the CprK homologues. CRP, cAMP binding protein from *E. coli*; FNR, fumarate and nitrate respiration regulator from *E. coli*; CprK, transcriptional regulator of halorespiration from *D. dehalogenans*; CprK1-K5, known or putative transcriptional regulators of halorespiration from *D. hafniense*.

In the recognition α-helixes (α_F) of the HTH domain, CprK1 and CprK2 possess a conserved motif V--SR (Fig. 5.2); in CprK4 the corresponding sequence is V--SK, whereas in CprK3 and CprK5 it is V--CK. The position of these residues corresponds to the motif E--SR in *E. coli* FNR, which has been demonstrated to be responsible for the specificity towards its target DNA sequence TTGAT-N_4-ATCAA (FNR-box).

Based on the high homology of the CprK-paralogues to characterized CprK transcriptional regulators as well as on their chromosomal position, a search has been performed for regulator binding motifs in the upstream sequences of all the *cpr* genes. Ten putative regulatory sequences, termed dehaloboxes (DB1-10), were identified using the MEME algorithm (Bailey *et al.*, 1994); in addition, one distinctly related motif was found in the promoter region of the putative 3,5-DCP reductive dehalogenase-encoding gene *cprBA5* (DB0) (Fig. 5.1a,b). The identified sequences included three dehaloboxes that were previously studied in detail (Gábor *et al.*, 2006). Each dehalobox contains a 5-bp non perfect inverted repeat with 4 nucleotide spacing, and the consensus sequence of dehaloboxes is TTAGT-N_4-ACTAA (Fig. 5.1b).

Transcriptional activation by CprK-paralogues in E. coli

In order to study the nature of the putative CprK-binding motifs (dehaloboxes) that were found upstream of the *cpr* genes (Fig. 5.1a,b), a recombinant *in vivo* promoter-probe assay was developed in *E. coli*. Each *cprK*-encoding gene was PCR-amplified from *D. hafniense* genomic DNA, cloned into pET24d expression vector and used to transform *E. coli* JM109(DE3) host cells. After optimisation of the culturing conditions for protein synthesis, CprK1 (26.5 kDa), CprK2 (26.5 kDa) and CprK4 (27 kDa) were successfully overproduced in *E. coli*, partly in the soluble fraction (Fig. 5.1c). Despite of repeated efforts, the soluble overproduction of CprK3 and CprK5 was not achieved. CprK1 appeared as a double band during SDS PAGE analysis (Fig. 5.1c); mass spectrometry measurements have demonstrated that this (at least in part) is due to removal of the N-terminal methionine (Chapter 4 of this thesis). A promoterless β-galactosidase-encoding gene was fused with *D. hafniense* promoter fragments, each containing a dehalobox centred in the middle of a 0.2-kb DNA fragment. The resulting promoter-probe constructs (pWUR216-223) are listed in Table 5.1.

We tested the *in vivo* transcriptional activation within gene clusters by co-transforming *E. coli* JM109(DE3) host cells with the CprK2-encoding plasmid (pWUR227) and dehaloboxes from its own gene cluster (DB1-DB4); or the CprK4-encoding plasmid (pWUR226) and dehaloboxes DB8-DB10. Triplicate cultures of each promoter-fusion containing strains were grown under two conditions: in the presence of 20 mM CHPA as effector compound, or in the absence of the halogenated compound. The functionality of CprK2 and CprK4 on the respective promoters were analysed by detecting the activity of the β-galactosidase reporter enzyme (Fig. 5.3), and data were compared with results that had previously been obtained for CprK1 on DB5-DB7 (Gábor *et al.*, 2006).

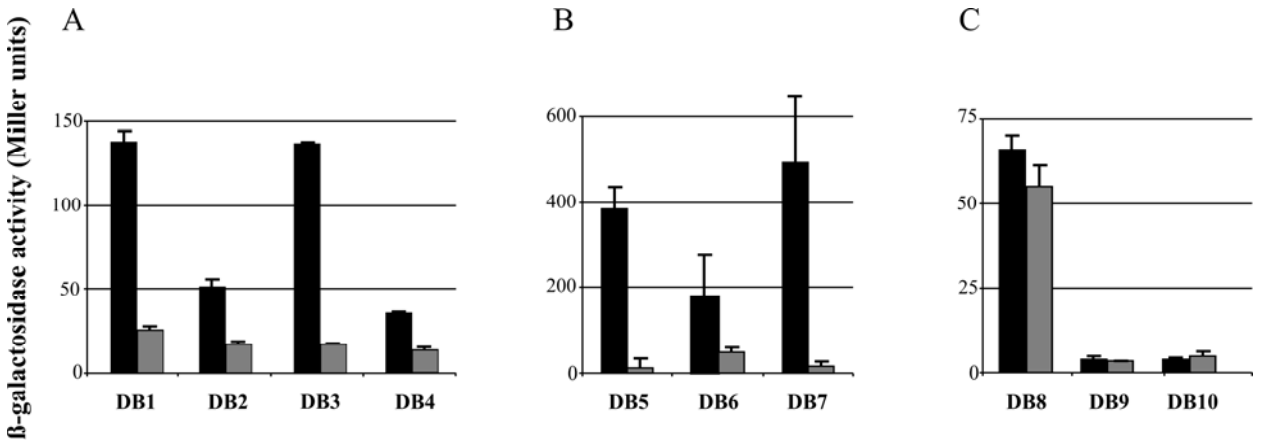

Figure 5.3 *In vivo* promoter probe assays using recombinant *E. coli* JM109(DE3) cells overproducing CprK2 (**A**), CprK1 (**B**) or CprK4 (**C**). Dehaloboxes (DB1-10) centred in *D. hafniense* promoter fragments were fused to β-galacosidase-encoding genes and tested for transcriptional activation in the presence of 20 mM CHPA (solid bars) or in the absence of the effector (shaded bars). Results for Panel B were obtained previously (Gábor *et al.*, 2006), and are included for comparison.

CprK2 was most active on the promoter that contains dehalobox DB1 and is situated upstream of the putative reductive dehalogenase-encoding gene *cprBA2* (Fig. 5.3a). Similar activity was measured on the promoter of *cprK3*, containing DB3. The putative promoters that contain DB2 and DB4 dehaloboxes were weaker, showing approximately 40% residual activity.

Remarkably, transcriptional activation by CprK4 was only observed with DB8, a dehalobox that is situated upstream of a putative methyl-accepting protein-encoding gene (*macA*) (Fig. 5.3c). The fact that *macA* coding for an important player of the chemotaxis machinery is upregulated under halorespiring conditions, might suggest that *D. hafniense* elicits chemotactic response to chlorinated compounds. Promoter activity mediated by CprK4 from DB9 or DB10 (upstream *cprK4* and *cprBA4*, respectively) was negligible. This indicates that the transcription of *cprK4* and *cprBA4* might be activated by a different regulator, or alternatively, their transcription can be initiated by CprK4 from *macA* by readthrough. One important difference was observed in the pattern of transcriptional activation by CprK-like proteins: while CprK1 (Fig. 5.3b) and CprK2 (Fig. 5.3a) were strongly regulated by CHPA, meaning that the promoter activity is drastically reduced when this compound is absent from the media, CprK4 is equally active with or without CHPA (Fig. 5.3c). This matter is further discussed below, during evaluation of the *in vitro* electrophoretic mobility shift experiments.

Dehaloboxes: consensus or optimal DNA sequence?

The *in vivo* promoter-probe experiments showed that similarly to CprK1, the two new CprK paralogues (CprK2 and CprK4) are also involved in the recruitment of RNA polymerase and consequently in transcriptional activation in *E. coli* (Fig. 5.3). All the *D. hafniense* promoter fragments that were used in the assay harbour a 14-bp inverted repeat termed dehalobox as potential binding target for the CprK-paralogues. The consensus sequence TTAGT-N_4-ACTAA for dehaloboxes was derived from a sequence alignment (Fig. 5.1b). Interestingly, all dehalobox sequences show deviations from this consensus sequence. Also, CHPA-induced promoter activities varied by two orders of magnitude, while only a 2-3 fold variation was observed in the yield of functional (i.e. soluble) production of CprK-homologues by *E. coli* JM109(DE3) (Fig. 5.1c). Hence, the difference in protein concentration does not explain the observed 100-fold difference in the promoter activities. The possible effect of deviations from the consensus dehalobox sequence (TTAGT-N_4-ACTAA) was studied by computing the correlation coefficient on promoter activity. Correlation was measured on a 0 to 1 scale, where values up to 0.4 show negligible to weak correlation, while values above 0.7 indicate a strong to very strong correlation between two variables. Interestingly, only a very weak (-0.18) negative correlation was measured between the activity and the degree of discrepancy from the consensus sequence. Similar results were obtained, when changes in the consensus Pribnow sequence (TATAAT) were also taken into account (-0.25 correlation coefficient). This questioned the accuracy of the determined consensus sequence and prompted us to look further for an optimal dehalobox sequence.

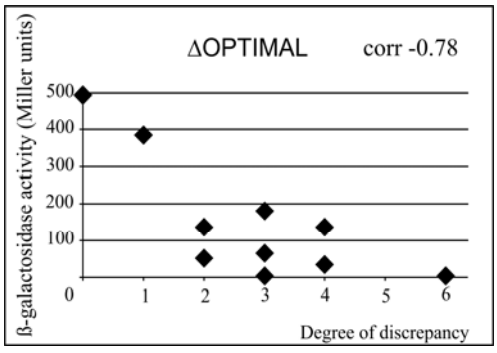

Figure 5.4 Correlation between promoter activity mediated by the CprK-paralogues and the degree of discrepancy from the optimal regulator binding sequence. Promoter activity is expressed in Miller units (enzymatic activity of the reporter protein); the degree of discrepancy is given as the number of nucleotides that differ from the TTAAT-N_4-ATTAA optimal dehalobox sequence.

Among the ten studied dehaloboxes, only DB7 is a perfect inverted repeat (TTAAT-N_4-ATTAA). In addition, it is notable that among all the promoters, that of *cprBA1* containing DB7 shows the highest promoter activity, not only in the presence of CprK1 (500 Miller units; Fig. 5.3b) but also with CprK4 (130 Miller units; not shown). This makes DB7 a candidate optimal binding target for CprK-like proteins. Indeed, a strong negative correlation (-0.78) was observed between the measured reporter enzyme activity and the degree of discrepancy from the putative optimal target sequence TTAAT-N_4-ATTAA (Fig. 5.4). Consequently, it is suggested that determination of the consensus sequence as binding target of a transcriptional regulator does not necessarily identify the most optimal binding site for the regulator.

Alternative effectors of CprK1 and CprK4

In vivo promoter-probe experiments in *E. coli* revealed an unusual feature of CprK4: although it did distinguish between preferred (DB8) and non-preferred (DB9 and DB10) *D. hafniense* promoter fragments, it was constantly active, irrespectively of the presence or absence of the potential effector CHPA (Fig. 5.3c). There are two possible explanations for this behaviour: either CprK4 was constantly activated by cAMP (a molecule that is present in *E. coli* but cannot be synthesised in *D. hafniense*) or alternatively, the effector-free CprK4 is already capable of DNA-binding and this ability is only enhanced in the presence of a true ligand molecule (apparently not by CHPA). In order to study the DNA-binding properties of CprK4 in the presence of different potential effector molecules, electrophoretic mobility shift assays (EMSA) were performed. This *in vitro* method overcomes the difficulties raised by the toxic nature of these compounds when used in growth experiments. In parallel, the alternative effector-range of CprK1 was mapped with the same method. Attempts to functionally purify CprK2 were unsuccessful, due to the instability of the protein; therefore CprK2 was excluded from the EMSA experiments. Binding experiments were performed with small promoter fragments containing dehaloboxes for which the highest *in vivo* activity was measured previously (DB7 for CprK1 and DB8 for CprK4, Fig. 5.3). Labelled DNA fragments (1.5 to 2 nM) were incubated in the presence of 1 µM regulator protein and 800 µM potential effector molecules. These chemicals included cAMP, HPA (dechlorinated derivative of CHPA), di- and tri-chlorinated phenolic compounds that have been reported to be degraded by *D. hafniense* DCB-2 and/or the closely related *D. dehalogenans* (3,5-DCP, 2,3-DCP, 2,4-DCP, 2,4,5-TCP and 2,4,6-TCP) (Christiansen *et al.*, 1996; Utkin *et al.*, 1995), CHPA and its bromo- and fluoro-substituted derivatives (2-Br-4-CP and 2-F-4-CP). The experiments revealed that CprK4 is indeed capable of DNA-binding in its effector-free form (Fig. 5.5b), and that cAMP or CHPA does not promote DNA-binding to any extent. However, in the presence of compounds such as 3,5-DCP, 2,4-DCP and 2,4,6-TCP clearly more protein-DNA complexes were formed. It is interesting to note that exactly these compounds had none or very limited potential to act as effectors for CprK1 (Fig. 5.5a). This suggests a complementary function of the two paralogues. The analogues 2,4-DCP and CHPA are very good effectors for CprK1, similarly to 2-Br-4-CP.

The related compound that contained the more bulky fluor atom (2-F-4-CP) was a weak effector, similar to 2,4,5-TCP. Unexpectedly, in the presence of high molar excess of HPA, protein-DNA complexes were also formed. In the case of CprK4, addition of 2,4-DCP, 2-Br/F-4-CP and 2,4,5-TCP resulted in an intermediate level of increase in protein-DNA complex formation. In summary, the results indicate that a halogen substitution at the *meta* position in phenol-derivatives resulted in the loss of effector activity on CprK1; on the contrary, this appeared to be beneficial for the activity of CprK4 (Fig. 5.5c). The only restriction for CprK4 is that it cannot accept a long side-chain (like the acetic acid tail in CHPA) at the *para* position, probably due to steric constraints in the effector binding pocket. These results are in agreement with those obtained by μESI-MS (Chapter 4 of this thesis).

The last notable difference between the *in vitro* DNA-binding properties of the two proteins is the formation of a second, slower migrating protein-DNA complex with CprK4, which is not observed with CprK1 (Fig. 5.5b).

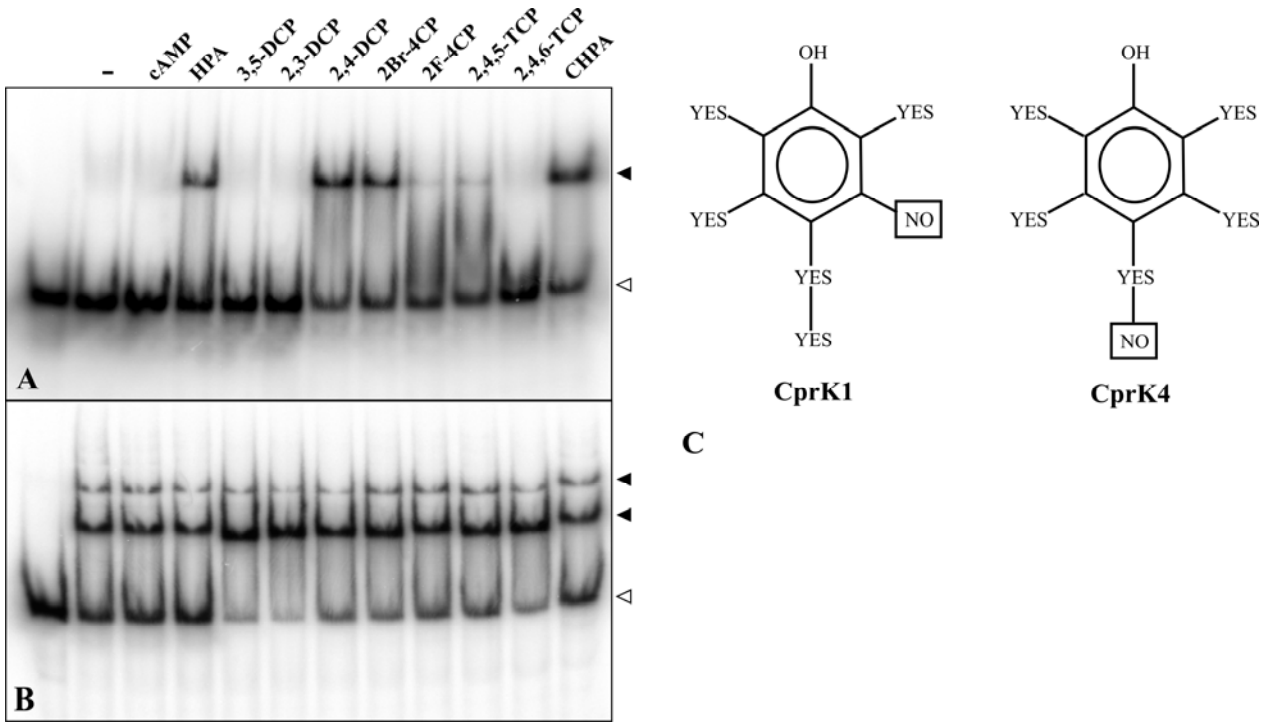

Figure 5.5 Alternative effectors of CprK1 and CprK4. EMSA was performed in the presence of halogenated compounds and compounds with related structure (as indicated above the image) and with constant amounts of CprK1 (**A**) or CprK4 (**B**). The reaction mixture contained dehalobox DB7 (CprK1) or DB8 (CprK4) and 800:1 molar ratios of effector and protein. The first lane in each gel served as a control, containing only labelled DNA. Free DNA is indicated with open arrowheads, protein-DNA complexes are marked with solid arrowheads. Panel (**C)** indicates the positions in phenol derivatives where substitutions are tolerated by CprK1 or CprK4.

This indicates that more CprK4 molecules are bound to the same DNA fragment, causing a further decrease in its mobility, either by forming a higher CprK4-oligomer (most likely tetramer, as proposed by Joyce et al., 2006) which then binds to the DNA, or by binding of a second CprK4-dimer to a weak, low-affinity DNA site. One weak inverted repeat (TTATC-N_4-ATTTA) that resembles the dehalobox consensus was indeed identified in a position that overlaps DB8. The possible, although fairly unlikely functionality of this putative binding site could give an explanation on how the CprK4-mediated transcriptional activation can be controlled by a co-operate action of a second transcriptional regulator. Alternatively, the expression of CprK4 itself could be controlled in a ligand-dependent manner, possibly by one of the other CprK homologues. This would be another example of hierarchy, which resembles how the expression of the constitutively active FixK is being turned on by FixLJ, an oxygen sensing two-component system (Batut *et al.*, 1989).

Discussion

The ongoing whole genome shotgun sequencing of *D. hafniense* DCB-2 genome revealed a redundancy in genes coding for potential reductive dehalogenases (*cprA*) and putative CRP/FNR-type transcriptional regulators (*cprK*), which enables this organism to gain energy from halorespiration (Smidt & de Vos, 2004; Villemur *et al.*, 2002). Moreover, these *cprA*- and *cprK*-like genes were found to be located in a conserved gene context resembling those found in other halorespiring bacteria (Smidt *et al.*, 2000). The work presented here focused on the diversity of predicted CprK regulators and aimed to gain more understanding on the following questions: (i) what is the role of multiple CprK-homologues in strain DCB-2 (overlapping or complementing function), (ii) how is their specificity exerted, or in other words, how is cross-talk of these highly similar regulators eliminated, and (iii) what is the origin of the redundancy of the CprK-like proteins.

We found that the CprK paralogues in *D. hafniense* DCB-2 have partly overlapping function as both CprK1 and CprK2 initiated transcription from dehalobox-containing promoters in the presence of CHPA. CprK1 and CprK2 are 62% similar at the amino acid level, which might indicate that the two regulators can substitute each other, and that they both control regulons with identical or at least overlapping functionality. Such interchangeable function has previously been demonstrated in *Lactococcus lactis*, where two closely related paralogues from the CRP-FNR family are present: FlpA and FlpB (75% similarity) encoded by distinct operons involved in metal ion transport. The single *L. lactis* Flp-mutants behaved similarly to the wild-type cells, only the double mutant showed an altered polypeptide profile and drastically decreased zinc-content (Gostick *et al.*, 1999). We also found that one CprK paralogue (CprK4) responds to a different set of effector molecules, indicating that it has a complementing function to CprK1 and CprK2. Specific DNA binding mediated by CprK4 was enhanced in the presence of *meta*-substituted compounds such as 3,5,-DCP and 2,3-DCP. On the contrary, these compounds did not induce DNA binding by CprK1.

The recently elucidated crystal structure of CprK1 revealed that such differences in effector specificity are expected, since among the CHPA-binding residues of CprK1, only Lys133 is conserved in all the CprK paralogues (CprK4 shares five identical effector-binding amino acids with CprK1) (Fig. 5.2) (Joyce *et al.*, 2006). In conclusion, we showed that CprK1, CprK2 and CprK4 can sense a range of halogenated phenol-derivatives in a co-operative action, with only partly overlapping specificity.

The large number of transcriptional regulators from the CRP-FNR family in the same organism raises the question of how target genes are discriminated in the simultaneous presence of these regulators. It has been speculated that subtle changes in the target DNA sequence, the exertion of hierarchical control and further protein requirements (co-activators and specific sigma factors) ensure the elimination of unwanted cross-talk of the similar regulators (Zumft, 1997). We showed that a strong negative correlation exists between deviations from the optimal CprK-binding sequence (TTAAT-N_4-ATTAA) and the promoter activity determined by using *lacZ* promoter fusions in *E. coli*. Conservation of the A/T and T/A base pairs at the symmetry related positions 3 and 12 in dehaloboxes ensures that these promoters are not recognised by proteins with FNR-type E--SR motif in their recognition α-helix (Gábor *et al.*, 2006). Previously it has also been demonstrated that both the spacing nucleotides of the inverted repeat and the nucleotides further downstream affect transcriptional activation, which enables fine tuning of promoter activity (Scott *et al.*, 2003; Veldman *et al.*, 2006). We did not observe a specific hierarchical control between the CprK paralogues from *D. hafniense*. However, as CprK4 showed a constitutive DNA-binding activity, it is possible that in *D. hafniense* the expression of *cprK4* is regulated in a hierarchical way, as described for FixK in *Rhizobium meliloti* (Batut *et al.*, 1989). The expression of *fixK* is activated in an oxygen-dependent manner by the two-component signal transduction proteins FixLJ. Interestingly, transposon mutagenesis studies in *D. dehalogenans* identified a mutant that was solely impaired in halorespiration due to the *Tn*916 insertion upstream of a putative sensory transduction histidine kinase-encoding gene (Smidt *et al.*, 1999). Another candidate hierarchical regulator is CprC, a putative membrane-bound NirI/NosR-type protein, the gene of which is often found to be clustered with twin-arginine signal peptide-encoding genes (Smidt & de Vos, 2004). Also, hierarchy can be exerted by means of other regulatory interactions. As an example, in *Paracoccus denitrificans* the role of three FNR-homologues (FnrP, NNR and NarR) is separated by the need for the presence of a specific sigma factor (Van Spanning *et al.*, 1997; Veldman *et al.*, 2006). However, in the CprK paralogues most of the characteristic negatively charged residues that make contact with the common σ^{70} factor of the *D. hafniense* RNA polymerase holoenzyme are conserved, thus it is unlikely that they utilize an alternative sigma factor (Gábor *et al.*, 2006). The presence of co-activators or repressors can also alter promoter activity (Barnard *et al.*, 2004; Bearson *et al.*, 2002). The results from gel retardation experiments indicate that the promoter regulated by CprK4 might contain one additional, weak affinity regulator binding site, as shown by the formation of a second, slower migrating band (Fig. 5.5b). This distinct complex might have deviating features, e.g. serving as target for a co-regulator.

This possibility would also explain how the unexpected constant promoter activity of CprK4 in *E. coli* might be attenuated in the host *D. hafniense*. Another possibility is that the physical shape of the DNA in complex with CprK4 is different under effector-free and effector-bound conditions, as has been shown for LysR-type regulators (Diaz *et al.*, 2000), which would enable RNAP recruitment only under halorespiring conditions.

Finally, we have demonstrated that at least three CprK paralogues are involved in sensing a range of halogenated compounds in *D. hafniense*. Genomic analyses have revealed that a large number of gene paralogues may be present in bacterial chromosomes. It is known that gene duplications and horizontal gene transfer (HGT) that give rise to gene paralogues play an important role in the evolution of bacterial genomes by providing a rapid means of adaptation to environmental changes (Hurles, 2004; Janssen *et al.*, 2005; Van der Meer *et al.*, 2003). Based on genome signature analysis, the halorespiring organism *Dehalococcides ethenogenes* strain 195 acquired most of its reductive dehalogenase-encoding genes (15 out of 18) via HGT (Regeard *et al.*, 2005). Similarly, it appears that reductive dehalogenase-encoding genes are associated with transposable elements and insertion sequences in *D. hafniense* strain TCE-1 (Maillard *et al.*, 2005). We found putative transposase-encoding genes in the genome of *D. hafniense* strain DCB-2, two in the close vicinity of the *macA-cprK*$_4$*BA*$_4$ and the *cprA*$_5$*B* reductive dehalogenase gene clusters. Remarkably, our results indicate that *macA*, a putative methyl-accepting chemotaxis protein-encoding gene is upregulated by CprK4 under halorespiring conditions. *D. hafniense* DCB-2 possesses one or two terminal flagella (Christiansen *et al.*, 1996) and with one exception (the phosphoprotein phosphatase *cheZ*) all the chemotaxis machinery *che* genes (the core-receptor-encoding *cheA*, *cheY* and *cheW*, and the modulating *cheR* and *cheB*) that would enable the bacterium to detect and respond to specific chemicals in the environment are present in the genome (Webre *et al.*, 2004). Thus it is possible that this particular CprK homologue regulates not only genes that are involved in degradation of haloaromatic compounds but also a gene that enables the organism to actively seek these compounds. Such a relationship has been described in the 4-hydroxybenzoate (4-HB) degrading *P. putida* PRS2000 strain between the PcaR transcriptional regulator and PcaK that is involved in chemotaxtic response to 4-HB (Harwood *et al.*, 1994), and for NahR and NahY from *P. putida* G7 for the degradation of and chemotaxis to the aromatic hydrocarbon naphtalene (Grimm *et al.*, 1999; Schell, 1985).

In conclusion, we investigated the role of multiple CprK paralogues in *D. hafniense* DCB-2, and found that two regulators (CprK1 and CprK2) have an overlapping effector-sensing function, while CprK4 shows distinct effector-characteristics. The DNA-binding specificity is possibly exerted by changes in the target nucleotide sequence from the consensus dehalobox sequence, and by additional regulator binding sites as proposed for the CprK4-regulated *macA* promoter. The redundancy of the *cprK* genes is likely to be caused by mobile genetic elements, as shown by the presence of putative transposase-encoding genes in the vicinity of the *cpr* gene clusters. The presence of a CHPA-inducible methyl-accepting chemotaxis protein-encoding gene in *D. hafniense* suggests that chemotaxis plays an important role in actively seeking halogenated compounds, which is a promising feature of this halorespiring bacterium for the use in bioremediation.

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Advances in overproduction and purification of active reductive dehalogenases from *Desulfitobacterium* spp.

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submitted

Abstract

Gaining knowledge of metalloenzymes that contain complex cofactors is often hindered by difficulties in producing soluble recombinant enzymes. By using molecular chaperons GroEL and GroES and a trigger factor, along with optimized culturing conditions, two cobalamin/iron-sulphur containing reductive dehalogenases from *Desulfitobacterium* spp. were successfully overproduced in *E. coli* and subsequently purified.

Introduction

Reductive dehalogenases are B_{12}-containing enzymes that catalyze the reductive removal of the halogen substituent from aromatic and aliphatic substrates by replacing it with hydrogen (hydrogenolysis) (Smidt & de Vos, 2004). They are able to dechlorinate several recalcitrant environmental pollutants, including the solvent tetrachloethene (PCE) (McCarty, 1997), chlorinated benzenes (Adrian *et al.*, 2000), polychlorinated biphenyls (PCBs) and their oxigenated metabolites, the hydroxylated PCBs (Krasotkina *et al.*, 2001). With one exception, all reductive dehalogenases (RD) contain B_{12} as cofactor, and majority of them also possesses two [4Fe-4S] and/or [3Fe-4S] redox centres. Photoreversible inactivation by 1-iodopropane indicated that the active site of the reductive dehalogenases is the super-reduced vitamin B_{12} (cob[I]alamin) (Wohlfarth *et al.*, 1997). However, the mechanism by which the electrons are transferred to the chlorinated substrate via cobalamin has not been elucidated yet. Two models have previously been proposed: one model presumes that an organocobalt adduct is formed prior to the carbon-halide bond cleavage, similarly to methyltransferases; according to the second model, however, a radical intermediate is involved in the dechlorination reaction, as has been described for B_{12}-dependent ribonucelotide reductases (Banerjee *et al.*, 2003).

In order to reveal the intriguing reaction mechanism, substantial quantities of wild-type and mutant proteins are required. Previously, two attempts were made for soluble and functional overproduction of recombinant RDs in *Escherichia coli*. A gene coding for a PCE reductive dehalogenase (PceA) from *Desulfitobacterium* sp. strain Y51 was cloned and overexpressed both aerobically and anaerobically in *E. coli* (Suyama *et al.*, 2002). However, PceA was produced in the insoluble fraction. With the help of a plasmid coding for a rare *E. coli* $tRNA^{Arg}$, Neumann *et al.* (1998) were able to overproduce PceA from *Dehalospirillum* (now: *Sulfurospirillum*) *multivorans* under aerobic conditions, yet the protein was non-functional.

The main bottleneck in the functional overproduction of metalloenzymes, and reductive dehalogenases in particular, is their notable oxygen-sensitivity ($t_{1/2}$ of 60 to100 min) and the need for correct assembly of the holoenzyme, including cofactor incorporation. The complex maturation pathway of RDs most likely involves the co-operate action of molecular chaperons and trigger factors that are encoded in the same gene clusters as the reductive dehalogenases (Smidt & de Vos, 2004; Villemur *et al.*, 2002).

Recently, it has been reported that co-expression of GroEL/ES chaperons and the TF trigger factor from a plasmid pG-Tf2 resulted in increased soluble overproduction of several proteins using *E. coli* as a host (Nishihara *et al.*, 2000). In *E. coli,* GroEL together with GroES is involved in assisting the correct folding of newly synthesized proteins, as well as they permit misfolded proteins to unfold and refold (Fink, 1999). The effect of GroEL was shown to be enhanced by the interaction with the peptidyl-prolyl *cis/trans* isomerase TF (Kandror *et al.*, 1997).

The aim of our study was to investigate the effect of GroEL/ES and TF co-expression in combination with improved culturing conditions on the recombinant production of two *ortho*-chlorophenol reductive dehalogenases (CprA) from *Desulfitobacterium* spp.

Materials and Methods

Cloning of the reductive dehalogenase-encoding genes

The *cprA* gene (without the coding region for the Tat signal peptide) was PCR amplified from *Desulfitobacterium dehalogenans* genomic DNA using oligonucleotide primers BG1242 (5'-GCGCGCCATGGCTGAAACAATGAACTATGTCCCG-3') and BG1433 (5'-GCGC GGGATCCATAGGTTTCAAGGGATACTTTTCAGG-3') that introduced an *Nco*I and a *Bam*HI restriction site at the 5' and the 3' end, respectively (underlined nucleotides). The resulting PCR product was digested and ligated into a linearized pET24d vector (Novagen), in frame with a C-terminal 6×His translational fusion, resulting in plasmid pWUR130. A similar approach was used for the cloning of the leaderless *cprA4* gene: BG1434 (5'-GCGGTCATGATAAACA ACTATGAGTAC-3') and BG1435 (5'-GCGCCTCGAGATAATCGAATTTATACAATTC-3') oligonucleotide primers introduced a *Bsp*HI and an *Xho*I restriction site in the PCR product amplified from *Desulfitobacterium hafniense* genomic DNA, which was subsequently ligated into an *Nco*I/*Xho*I-digested pET24d vector. The resulting pWUR131 plasmid contained *cprA4* with a C-terminal 6×His translational fusion.

Overproduction of CprA and CprA4 reductive dehalogenases

A colony of freshly transformed *E. coli* BL21(DE3) harbouring pG-Tf2 (Nishihara *et al.*, 2000) and either pWUR130 or pWUR131 was grown overnight (O/N) aerobically at 37°C in Luria-Bertani (LB) medium supplemented with kanamycin (30 μg/ml) and chloramphenicol (35 μg/ml). All the subsequent steps were done anaerobically, using media and stock solutions that were flushed (3×) with N_2. LB medium (2 litres) was inoculated with 5% O/N culture and supplemented with glucose (0.4%), fumarate (20 mM), vitamin B_{12} (50 μg/L), Fe(III)-citrate (50 μM), Fe(II)-ammonium sulphate (50 μM), kanamycin (30 μg/ml) and chloramphenicol (35 μg/ml). Finally, the medium was reduced by adding 75 μM Ti(III) citrate (Zehnder *et al.*, 1976). Production of chaperons from the pG-Tf2 plasmid and the recombinant RDs from the pET24d derivatives was induced by tetracycline (15 ng/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM), respectively.

The induction temperature was optimized as described in the Results and Discussion session. IPTG was supplied at OD_{600} 0.3, while tetracycline was added at the time of inoculation (pWUR131) or at the time of IPTG induction (pWUR130).

Purification of CprA and CprA4 by affinity chromatography

Recombinant cells were harvested under anoxic conditions by centrifugation, and transferred into an anaerobic glove box with N_2/H_2 (96:4 v/v) as a gas phase. All subsequent purification steps were done anaerobically. The cell pellet was resuspended in buffer L (50mM NaH_2PO_4 [pH 8.0], 300mM NaCl), disrupted by sonication (5 × 15 sec, with 20 sec intervals on ice) and centrifuged (16, 000 × g, 10 min, RT) to remove cell debris. After loading 250 µl chelating sepharose (Amersham Biosciences) in a Bio-Spin chromatography column (Bio-Rad), the column was washed with 5 × the column volume (CV) of demineralized water, loaded with 0.1 M $NiCl_2$ (1×CV), and washed with demineralized water (2×CV). The column was equilibrated with 4×CV buffer LX (50mM NaH_2PO_4 [pH 8.0], 300mM NaCl, 2% Triton X-100, 20% glycerol), cell free extract was then loaded on the column, gently mixed and incubated for 30 min at RT. Cell free extract of CprA contained 0.8 mM DTT for improved binding. Weakly bound contaminant proteins were washed by applying 1×CV buffer W1 (buffer LX + 10 mM imidazole) followed by 2 CV buffer W2 (buffer LX + 20 mM imidazole). Finally, 6×His tagged reductive dehalogenases were eluted by applying 0.5 CV buffer LX that contained 250/500/1000 mM imidazole. Eluted fractions were desalted and concentrated using Centriplus 10,000-molecular-weight-cutoff centrifugal devices (Amicon).

Immunoblot assay

The Western blot assay was performed using Penta-His antibodies (Qiagen) according to the protocol of the antibody's supplier, with the following changes. After denaturing SDS-PAGE gel electrophoresis, the protein samples were transferred to a nitrocellulose membrane in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer (pH 11.0) containing 10% methanol. Overnight electroblotting at 70 mA was done in a cooled Bio-Rad Mini Trans-Blot tank. In order to reduce non-specific background signals, TBS buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl) was supplemented with 3% BSA in all membrane washing steps. After incubation with the primary mouse antibody (1000 times dilution), chromogenic immunodetection was done with a goat Anti-Mouse IgG AP-conjugate (Promega) in 7500 times dilution.

Reductive dehalogenase activity assay

The purified and concentrated protein samples were tested for activity according to a previously established protocol (Van de Pas *et al.*, 1999). The reaction mixture was prepared in a N_2-flushed 1-cm cuvette, which contained 50 mM Tris-HCl (pH 7.8) and 0.3 mM methyl viologen, and was incubated at 30°C.

The artificial electron donor methyl viologen was reduced by titanium(III) citrate to a stable absorption ($A_{578} \cong 2.2$). Then the enzyme preparation was added, and the assay was started by the addition of 3-chloro-4-hydroxyphenylacetic acid (CHPA) to 1 mM final concentration. One unit was defined as the amount of enzyme that catalyzes the reduction of 1 µmol of CHPA or the oxidation of 2 µmol of reduced methyl viologen per minute.

Results and Discussion

Reductive dehalogenase-encoding genes from Desulfitobacteria

Two *ortho*-chlorophenol reductive dehalogenases were selected for overproduction in *E. coli*: CprA from *Desulfitobacterium dehalogenans* (Van de Pas *et al.*, 1999), and CprA4 (72% sequence similarity to CprA) that was recently identified by homology search in the genome of the closely related *Desulfitobacterium hafniense* DCB-2 (Villemur *et al.*, 2002). The gene coding for CprA in *D. dehalogenans* is present in the *c*hloro*p*henol *r*eductive dehalogenase (*cpr)* gene cluster, which consists of *cprTKZEBACD* open reading frames (Fig. 6.1a) (Smidt *et al.*, 2000). Among the genes, the highly hydrophobic product of *cprB* is predicted to be a membrane-anchor for CprA, *cprC* and *cprK* encode putative or characterised NosR/NirI-type and CRP/FNR-type transcriptional regulators, respectively (Gábor *et al.*, 2006; Pop *et al.*, 2004). There are three putative protein folding catalysts encoded in the *cpr* gene cluster: the products of *cprD* and *cprE* show homology to GroEL-type chaperons, while *cprT* probably encodes a trigger factor (proline *cis/trans* isomerase). Besides the corresponding *cprB* and *cprK* genes, a putative methyl-accepting chemotaxis protein-encoding gene (*macA*) is also present in the *cprA4* gene cluster of *D. hafniense* (Fig. 6.1b). Both reductive dehalogenases contain an N-terminal signal peptide (shown experimentally for CprA [Van de Pas *et al.*, 1999] or predicted for CprA4 using SignalP [Bendtsen *et al.*, 2004]) in which a distinctive twin-arginine sequence (S/T<u>RR</u>xFLK) is present (Fig. 6.1c). Proteins with this characteristic conserved motif often contain complex cofactors and are translocated through the bacterial membrane in their folded form (Berks *et al.*, 2000). Two iron-sulphur clusters are bound to CprA by a conserved ferredoxin-type cluster of cysteine residues (CxxCxxCxxxCP) (Bruschi *et al.*, 1988). Similarly to CprA, the second cluster in CprA4 lacks the first cysteine residue, therefore it is likely to bind a [3Fe-4S] cluster next to one [4Fe-4S] cluster. A typical bacterial B_{12}-binding motif (DxHxxG, Banerjee *et al.*, 2003) could not be identified either in CprA or in CprA4. CprA catalyzes the *ortho* dechlorination of several phenol derivatives (CHPA, 2-Br-4-CP, 2,3-DCP) (Van de Pas *et al.*, 1999). Due to the lack of biochemical characterisation data, the substrates of CprK4 are not known. However, its high homology to CprA (Fig. 6.1c) suggests that it might be capable of *ortho* dehalogenation of CHPA or related phenolic compounds.

Chaperon-aided overproduction of reductive dehalogenases

The genes encoding the mature CprA and CprA4 (thus without the coding sequence for the signal peptide) were cloned in a T7 promoter-based expression vector, pET24d. A freshly transformed colony of *E. coli* BL21(DE3) harbouring pG-Tf2 helper plasmid and either pWUR130 (CprA) or pWUR131 (CprA4) plasmids was used as an inoculation for an O/N aerobic preculture. The overproduction recombinant cultures were grown anaerobically in the presence of fumarate, and were supplemented with precursors for cofactor synthesis (ferrous and ferric iron sources and vitamin B_{12}).

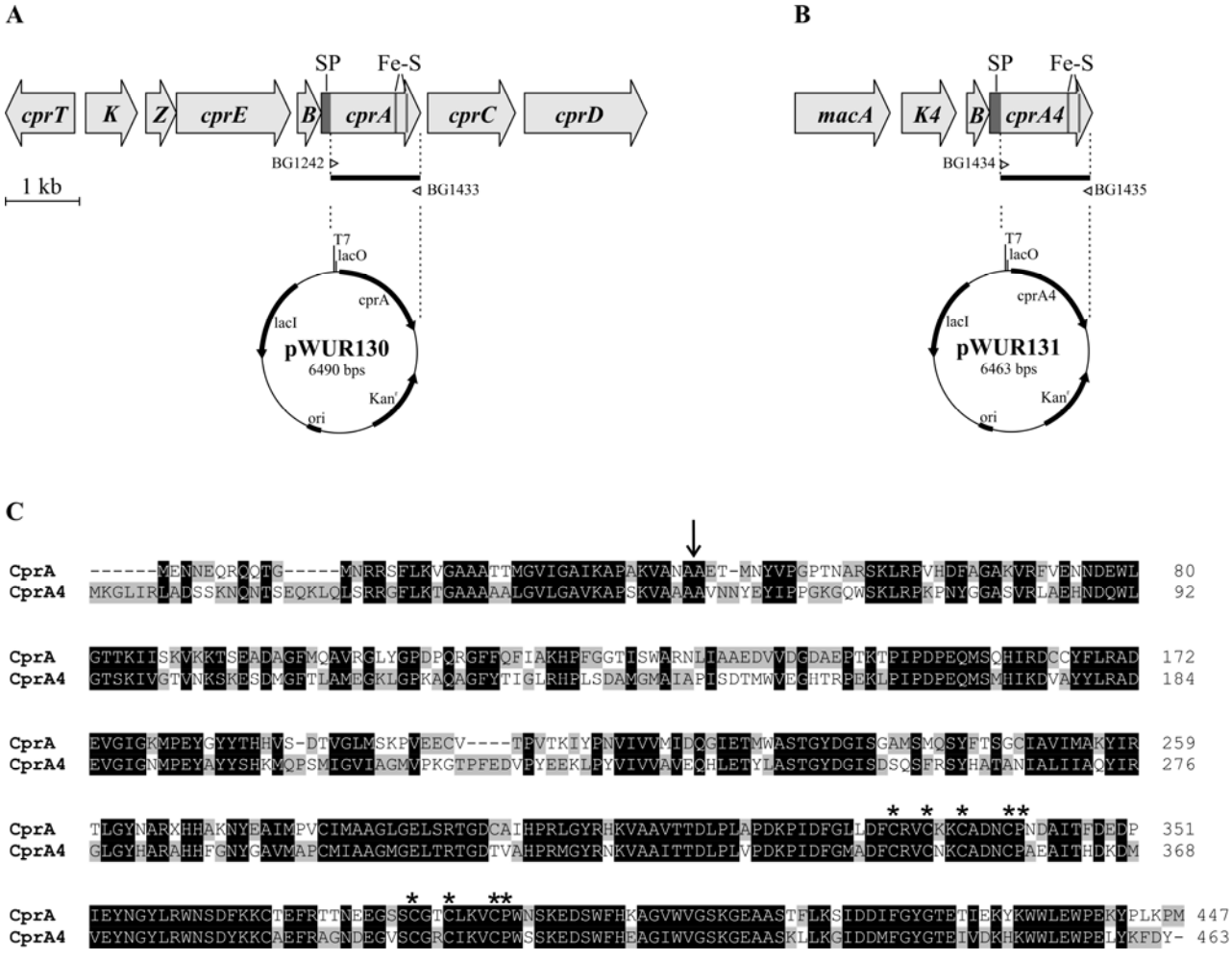

Figure 6.1 Chlorophenol reductive dehalogenase (*cpr*) gene clusters in *D. dehalogenans* (**A**) and in *D. hafniense* (**B**). The pWUR130 and pWUR131 expression vectors carry leaderless reductive dehalogenase-encoding genes: *cprA* (GeneBank Accession Number AF115542) or *cprA4* (GeneBank DhafDRAFT_0427, based on *D. hafniense* genome sequence version 24-Jun-2005). SP, twin-arginine-type signal peptide; Fe-S, conserved iron-sulphur binding motif. (**C**) Amino acid sequence alignment of CprA and CprA4. Signal peptide cleavage site is indicated with an arrow, the conserved ferredoxin-type clusters are indicated with asterisks.

When IPTG-induced protein production was conducted at 37°C in *E. coli*, all heterologously-produced CprA and CprA4 was found in inclusion bodies (data not shown). As lowering the induction temperature often results in the production of more soluble proteins due to the decreased rate of protein synthesis, the following changes were made: i) pre-induction growth temperature was lowered to 25°C or 30°C (pWUR130 or pWUR131 containing cells, respectively), and ii) after IPTG-induction, cultures were transferred to 15°C (pWUR130) or 20°C (pWUR131).

The synergistic action of the GroEL/ES chaperons, TF and the lowered induction temperature enabled 10-15% of the heterologously-produced protein to be synthesized in the soluble cell fraction (Fig. 6.2). Since the molecular size of the overproduced reductive dehalogenases (CprA 47.7 kDa, CprA4 46.8 kDa) overlaps with that of GroEL (48.0 kDa), these protein bands are not easily distinguishable from each other on Coomassie-stained SDS-PAGE gel (Fig. 6.2a). Therefore a Western blot was performed with the same protein samples using Penta-His antibodies, which specifically recognizes the 6×His tagged recombinant reductive dehalogenases. The results of the immunoblot assay confirmed that both CprA and CprA4 were overproduced, partly (10-15%) in the soluble fraction (Fig. 6.2b). The apparent additional low-molecular weight signal in CprA samples (lane 1 and lane 3) suggests possible proteolytic degradation.

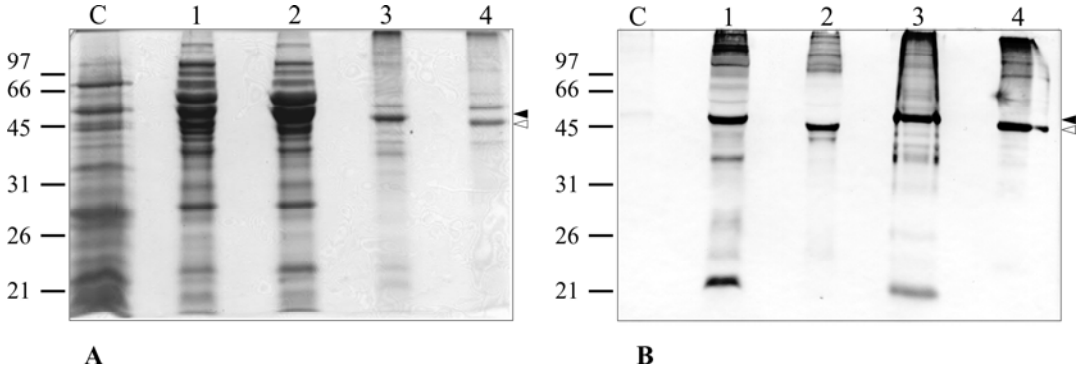

Figure 6.2 Overproduction of reductive dehalogenases in *E. coli*. (**A**) Coomassie-blue-stained 10% SDS PAGE gel showing different fractions of recombinant *E. coli* BL21(DE3)[pG-Tf2] cells after O/N induction. Cells were harbouring the following plasmids: C, pET24d (CFE, 1000× concentrated); 1, pWUR130 (CFE, 100× concentrated); 2, pWUR131 (CFE, 100× concentrated); 3, pWUR130 (I, 10× concentrated); 4, pWUR131 (I, 10× concentrated). CFE = soluble cell free extract, I = insoluble fraction. (**B**) Western blot assay of the same protein samples using anti-His-tag antibodies. Molecular size (kDa) of a broad range protein standard is indicated, positions of CprA and CprA4 are marked with a filled or open arrowhead, respectively.

Activity of purified heterologously-produced RDs

CprA and CprA4 were overproduced with a short affinity tag consisting of six histidines fused to their C-terminal end to facilitate purification by metal affinity chromatography. Our purification attempts, however, with the standard protocol using Ni^{2+}-nitrilotriacetic acid (NTA) spin columns (Qiagen) were unsuccessful, as most of the 6×His tagged protein failed to bind to the column matrix, irrespectively of the anaerobic or aerobic conditions we used during purification (data not shown). Since the 6×His tag is undoubtedly present in the proteins (Fig. 6.2b), we tried to improve protein binding by changing the nickel-chelating matrix from NTA to chelating sepharose. Besides that, the conditions were also adjusted by including Triton X-100 and glycerol in all the buffers, as was used for the purification of the native CprA from *D. dehalogenans* (Van de Pas *et al.*, 1999). The changes improved binding of the recombinant proteins and helped efficient purification of CprA and CprA4 (Fig. 6.3). Apparently, it was also possible to remove the low-molecular weight degradation product of CprA (Fig. 6.2b), as indicated by the loss of the signal in Fig. 6.3a.

Eluted protein fractions were desalted to remove high concentrations of imidazole and concentrated by centrifugation, prior to activity measurements. As a positive control for the enzymatic assay, CHPA-grown *D. hafniense* cell extract was prepared as described previously (Van de Pas *et al.*, 1999). Cell free extracts (CFE) of recombinant *E. coli* BL21(DE3) cells that carried pG-Tf2 and an empty pET24d overexpression vector and were grown under the same inducing conditions as the overproduction strains, were used as negative control. The assay was conducted at 30°C, pH 7.8, using reduced methyl viologen as electron donor and the *ortho*-halogenated phenolic compound, CHPA as electron acceptor. The following activities were detected: CprA, 76 $\mu mol.ml^{-1}.min^{-1}$; CprA4 48 $\mu mol.ml^{-1}.min^{-1}$, with the negative control showing no background activity. The positive control CHPA induced *D. hafniense* cell extract showed significantly higher activity (1,101 $\mu mol.ml^{-1}.min^{-1}$).

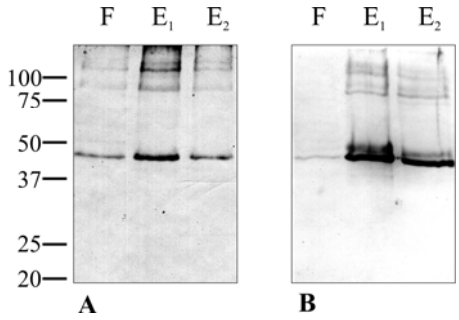

Figure 6.3 Purification of the heterologously overproduced reductive dehalogenases with Ni^{2+}-chelating sepharose affinity matrix. Western blots using anti-His-tag antibodies show different fractions from the purification: unbound samples (flow through, F) are followed by protein samples that were eluted from the column with 250 mM (E1) and 500 mM (E2) imidazole in the case of CprA (**A**) or with 500 mM (E1) and 1M (E2) imidazole in the case of CprA4 (**B**).

Conclusions

By co-expressing *E. coli* GroEL/ES chaperons and the trigger factor TF, we were able to produce soluble reductive dehalogenases and purify them to homogeneity. Enzymatic activity of the heterologously-produced enzymes was detected, however, it was significantly lower than that of the positive control (native enzyme preparation), which indicates that large portions of the non-native enzymes were likely lacking correctly incorporated cofactors. Recently it has been reported that the maturation process (cofactor incorporation, signal peptide processing) of the twin arginine (RR) signal peptide containing proteins (inc. reductive dehalogenases) is impaired when the RR-protein specific chaperons are replaced by non-specific chaperons (Palmer *et al.*, 2005). Therefore we suggest the following improvements: the replacement of GroEL/ES and TF with the possibly reductive dehalogenase-specific chaperons (CprD, CprE) and trigger factor (CprT) could result in much higher, likely active CprA production using *E. coli* as a host; thus in a protocol that would be beneficial for the overproduction of other reductive dehalogenases.

Acknowledgements

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7

Summary and concluding remarks

Halogenated organic compounds such as tetrachloroethene, dioxins and polychlorinated biphenyls have been widely used as biocides, intermediates in organic synthesis and in various industrial applications until recently. The inappropriate deposition of the hazardous halogenated organic waste poses a serious threat for the environment because of the toxicity and potential carcinogenicity of these compounds. A significant step towards dealing with halogenated hydrocarbons was the discovery of several strictly anaerobic bacteria that are able to couple the reductive removal of halogen atom(s) from these compounds – which often renders a less toxic or more bioavailable respiration end-product – to the generation of energy for bacterial growth by halorespiration. An important group among halorespiring bacteria is the genus *Desulfitobacterium* which includes low G+C gram-positive bacteria showing high versatility in their metabolic capability to degrade different halogenated aromatic and aliphatic compounds. *Desulfitobacterium dehalogenans* JW/IU-DC1 can reductively dechlorinate halogenated phenolic compounds at the *ortho* position, whereas *Desulfitobacterium hafniense* DCB-2 can dechlorinate not only *ortho*-substituted compounds but also 3,5-dichlorophenol at the *meta* position. The dechlorination reaction is catalysed by reductive dehalogenases, enzymes that contain a cobalamin cofactor and iron-sulphur redox centres. These enzymes form a new group within the cobalamin-containing enzymes, with a yet-unidentified reaction mechanism by which the electrons are transferred to the halogenated substrate via cobalamin. The majority of the reductive dehalogenases are only produced by halorespiring bacteria when a substrate halogenated hydrocarbon is present in their environment. This suggests a mechanism which enables these organisms to sense the presence of such compounds and to respond to them by activating the transcription of genes that code for proteins involved in the catabolism of organohalides. The research described in this thesis aimed to reveal details of the molecular basis of halorespiration in two main aspects: i) on the reaction mechanism that underlies the carbon-halide bond cleavage in reductive dehalogenases; and ii) on identifying the main factors (regulator protein, target DNA and effector compounds) that are essential for the activation of genes involved in halorespiration.

In **Chapter 1** a general overview is given on our present knowledge about halorespiration, and on closely related subjects. These include the natural and anthropogenic production of halogenated hydrocarbons, and their impact on biological systems, the diversity of halorespiring bacteria that can degrade organohalides by means of specific catalysts, the reductive dehalogenases, for which a comprehensive overview and two models that describe their possible reaction mechanism are presented. Also, known or putative transcriptional regulators of halorespiration and their homologues, particularly the cAMP receptor protein (CRP) and the fumarate nitrate reduction regulator (FNR) of *Escherichia coli* are introduced. Special attention is given to aspects of the structure-function relationship in CRP/FNR-type transcriptional regulators and to the complex, multilevel control of metabolic processes in bacteria. Finally, the emerging field of genomics of halorespiring bacteria is presented based on bioinformatics studies on the genome of four halorespiring bacteria.

Chapter 2 describes a new member of the CRP-FNR family, CprK1, a transcriptional regulator which activates expression of a set of genes that are involved in halorespiration in *D. hafniense*.

To investigate the interaction of CprK1 with three of its predicted binding sequences that were identified as 14-bp inverted repeats (dehaloboxes) in halorespiration-induced promoters, *in vitro* DNA-binding assays as well as *in vivo* promoter probe assays were performed. It was demonstrated that CprK1 binds its target dehaloboxes (consensus TTAAT----ATTAA) with high affinity in the presence of 3-chloro-4-hydroxyphenylacetic acid (CHPA), and not with its dechlorinated derivative HPA. A mutant CprK1 protein was created by a Val→Glu substitution at a conserved position in the DNA-binding recognition α-helix, in an attempt to engineer its binding specificity. Indeed, the mutant gained an FNR-type DNA-binding specificity, recognising the sequence TTGAT----ATCAA (FNR-box) instead of dehaloboxes, giving further insight on the molecular basis of the interaction between target DNA and transcriptional regulators. CprK1 was subject to oxidative inactivation *in vitro,* most likely caused by the formation of an intermolecular disulfide bridge between Cys11 and Cys200. The possibility of redox regulation of CprK1 by a thiol-disulfide exchange reaction was investigated by the construction and characterization of two Cys→Ser mutant proteins. Our results indicated that although the formation of Cys11-Cys200 disulfide bridge inactivates the protein *in vitro*, it does not appear to play a physiological role in the regulation of CprK1 *in vivo*.

Although many details of transcriptional activation by CprK1 were revealed by means of DNA-binding assays and promoter probe constructs, yet several interesting questions remained unanswered: how can the regulator so accurately distinguish the effector CHPA from its dechlorinated, biologically inactive derivative, HPA? What are the allosteric effects that enable CprK1 to recognize and bind its specific DNA target upon CHPA-binding? These questions are addressed in **Chapter 3**, which describes the 2.2 Å crystal structure of the oxidized CprK1 in complex with CHPA, together with the 2.8 Å crystal structure of the highly related CprK from *D. dehalogenans* in the ligand-free, reduced state. The structures revealed that dimeric proteins are folded into three distinct domains: an N-terminal effector-binding β-barrel, a long dimerization α-helix and a C-terminal DNA-binding domain with a common helix-turn-helix motif. Binding of CHPA induces structural changes in the dimerization α-helix where the chlorine atom binding residues Tyr130 and Leu131 are located. Besides the lack of these interactions with the dechlorinated derivative HPA, another important factor ensures the CHPA-specific allosteric transition of CprK1. A conserved lysine residue (Lys133 in CprK1) makes an essential bond with the phenolate group of CHPA. At physiological pH, this hydroxyl group is in the protonated form in HPA, and therefore a crucial bond which is involved in the repositioning of the N-terminal β-barrel is not formed ("pK_a interrogation" theory). Besides these differences, an unexpected dimerization of the DNA-binding α-helixes was observed in the structure of the effector-free CprK. It has been postulated that the ligand-induced conformational changes (which involves repositioning of β strands 4 and 5) destabilize the dimerization of the C-terminal domains and therefore allow the formation of new interdomain contacts. This can then lead to reorientation of the helix-turn-helix motif into its correct DNA-binding conformation.

In **Chapter 4** we report on the structural dynamics and ligand-induced conformational changes of CprK1 studied by macromolecular native mass spectrometry, limited proteolysis and *in vitro*

DNA-binding assays. A small library of potential effector molecules known to be dehalogenated by *D. hafniense* DCB-2 or *D. dehalogenans* were used in these experiments. Under reducing conditions, strong effector molecules (CHPA; 2,4,5-trichlorophenol; 2-Br-4-chlorophenol; 2,4-dichlorophenol) induced complex formation with the dehalobox DNA. Phenol derivatives that did not contain a halogen moiety at the *ortho* position and an acetate or halogen substitution at the *para* position (i.e. HPA; 2,3-dichlorophenol and 3,5-dichlorophenol) did not cause an allosteric transition on CprK1. Also, oxidized CprK1 – containing an intermolecular disulphide bridge between Cys11 of one monomer and Cys200 of the other – was not capable of DNA binding. Thus it was concluded that both reduction of the protein and the binding of a suitable phenol derivative (with halogen and/or acetate substitution at *ortho* and *para* position) are required for promoting the conformational change that leads to DNA binding. The changes in the structural dynamics of CprK1 upon reduction and due to effector and DNA binding were investigated by limited proteolysis coupled to mass spectrometry. Generally, the protein became more flexible upon reduction, as shown by a two-fold increase of rapid cleavages compared to oxidizing conditions. The binding of strong effector molecules induced changes in the dynamics of the sensory module of CprK1, while the DNA-binding domain overall remained to be sensitive to protease digestion, irrespective of the absence or presence of effectors. Binding of DNA was shown by the protection of a 14-amino acid polypeptide which contained the V--SR conserved motif known to be involved in specific DNA recognition (described in Chapter 2).

In the previous chapters, one particular transcriptional regulator, CprK1 of *D. hafniense* was in the focus of the investigations. However, in the partially sequenced genome of *D. hafniense* four additional genes are present that encode a CprK paralogue, three of which are clustered with potential reductive dehalogenase-encoding genes. In **Chapter 5** the role of multiple CprK paralogues from *D. hafniense* was studied by using an *in vivo* promoter probe assay in *E. coli* and *in vitro* DNA-binding assays. The results showed that two regulators (CprK1 and CprK2) have an overlapping effector-specificity with preference for an *ortho*-chlorophenol (CHPA), while the effectors of CprK4 included *meta*-chlorophenols (2,3-dichlorophenol and 3,5-dichlorophenol). The presence of two potential transposase-encoding genes in the vicinity of the *cprK* genes indicated that their redundancy is probably caused by mobile genetic elements. The CprK-paralogues activated transcription from promoters containing a 14-bp inverted repeat (dehalobox) resembling the FNR-box. We found a strong negative correlation between the rate of transcriptional activation and discrepancies of the target sequence from the optimal regulator-binding site. A strong halorespiration-inducible promoter was found upstream a gene that encodes a methyl-accepting chemotaxis protein, which is the first indication for taxis of an anaerobic bacterium to halogenated aromatic compounds.

Finally, **Chapter 6** describes the first step towards the ultimate goal, i.e. the unraveling of the novel reaction mechanism of reductive dehalogenases. In order to study the exact role of the cobalamin cofactor and the iron-sulphur clusters, and to identify the ligands that bind cobalamin (a common B_{12}-binding motif is not present in the protein), substantial quantities of wild-type and mutant proteins are required. However, production of metalloenzymes, and reductive

dehalogenases in particular, has so far been hindered by difficulties in obtaining soluble recombinant enzymes, mainly originating from the notable oxygen sensitivity of these enzymes and the need for correct cofactor incorporation into the protein. Therefore a practical approach is described here for the heterologous overproduction of two cobalamin/iron-sulphur containing reductive dehalogenases from *Desulfitobacterium* spp. by co-expression of protein folding catalysts to aid functional protein synthesis. We showed that simultaneous production of the molecular chaperons GroEL/ES and a trigger factor (TF), together with optimized anaerobic culturing conditions, resulted in overproduction of the reductive dehalogenases, partly (10-15%) in the soluble cell fraction of *E. coli*. Enzymatic activity of the recombinant enzymes was detected, however, it was significantly lower than that of the positive control (native enzyme preparation), which indicates that large portion of the heterologously-produced enzymes was probably lacking the correctly incorporated cofactors. Based on recent findings, we suggested that replacement of GroEL/ES and TF with the possibly reductive dehalogenase-specific chaperons (CprD, CprE) and trigger factor (CprT) could result in much higher, likely active CprA production in *E. coli*; thus in a protocol that would be also beneficial for the overproduction of other reductive dehalogenases.

In conclusion, our research on the transcriptional regulation and the catalytic basis of halorespiration contributes to the better understanding of the bacterial ability to degrade mainly man-made environmental pollutants, also of the factors that influence bacterial response to a certain group of these pollutants (halogenated hydrocarbons), and on how bacteria are continuously evolving these abilities. This research contributes to the development of a new, increasingly recognised concept in environmental biotechnology: instead of aiming at the design of improved bioremediation catalysts and "super-bugs" in the test tube by genetic engineering, scientists attempt to understand how bacteria respond to environmental signals (such as halogenated pollutants) and how this response is integrated into the global regulatory network within the individual bacteria and the entire community[1]. By understanding the conditions that affect the emergence and expression of desired metabolic activities within the indigenous community, it may be possible to stimulate bacterial degradation of environmental pollutants by eco-engineering.

1. Cases I, de Lorenzo V: Genetically modified organisms for the environment: stories of success and failure and what we have learned from them. Int. Microbiol. 8:213-22, 2005

8

Samenvatting en conclusies

Gábor, Krisztina – Moleculaire analyse van halorespiratie in *Desulfitobacterium* spp.: katalyse en transcriptionele regulatie.

Organische halogeen-verbindingen zoals tetrachlooretheen, dioxines en polychloorbifenylen (PCBs) werden tot voor kort nog veelvuldig gebruikt als biociden, intermediaire verbindingen in de organische synthese en in verschillende industriële toepassingen. Ondoordacht lozen van het gevaarlijke gehalogeneerd organisch afval vormt een ernstige bedreiging voor het milieu, vanwege de toxiciteit en potentiële carcinogeniteit van deze verbindingen. Heel belangrijk voor het verwijderen van gehalogeneerde koolwaterstoffen was de ontdekking van enkele strikt-anaërobe bacteriën die in staat zijn tot de reductieve omzetting van deze verbindingen, waarbij vaak minder toxische of beter afbreekbare ademhalingseindproducten ontstaan; omdat dergelijke omzettingen energie opleveren, leidt het proces van halorespiratie tot bacteriële groei. Een belangrijke groep van dehalorespirerende bacteriën is het geslacht *Desulfitobacterium*; dit zijn laag G+C gram-positieve bacteriën met een sterk uiteenlopende metabole capaciteit voor wat betreft de afbraak van verschillende aromatische en alifatische halogeenverbindingen. *Desulfitobacterium dehalogenans* JW/IU-DC1 kan fenol-achtige halogeenverbindingen reductief dechloreren op de *ortho*-positie, terwijl *Desulfitobacterium hafniense* DCB-2 niet alleen *ortho*-gesubstitueerde verbindingen maar ook 3,5-dichloorfenol op de *meta*-positie kan dechloreren. De dechloreringsreactie wordt gekatalyseerd door enzymen (reductieve dehalogenases) die een cobalamine cofactor en ijzer-zwavel redoxcentra bevatten. Deze dehalogenasen vormen een nieuwe groep binnen de cobalamine-bevattende enzymen, met een nog onbekend mechanisme om elektronen via cobalamine te transporteren naar het gehalogeneerde substraat. Het merendeel van de reductieve dehalogenases worden alleen geproduceerd door de halorespirerende bacteriën als er een gehalogeneerde koolwaterstof als substraat aanwezig is in het milieu. Dit duidt op een mechanisme dat deze organismen in staat stelt om de aanwezigheid van dergelijke verbindingen waar te nemen, en daar vervolgens op te reageren door activering van transcriptie van genen die coderen voor eiwitten die betrokken zijn bij het katabolisme van organohalogeen-verbindingen. Het onderzoek beschreven in dit proefschrift had als doel om details te ontrafelen van de moleculaire basis van halorespiratie met betrekking tot twee hoofdpunten: i) het reactiemechanisme van reductieve dehalogenases dat verantwoordelijk is voor het verbreken van de koolstof-halide binding; en ii) het identificeren van de belangrijkste factoren (regulatie-eiwitten, DNA-eiwit interactie, en effector verbindingen) die essentieel zijn voor de activering van genen die betrokken zijn bij halorespiratie.

In **Hoofdstuk 1** wordt een overzicht gegeven van de huidige kennis over halorespiratie, en daaraan sterk gerelateerde onderwerpen. Dit omvat de natuurlijke en antropogene productie van gehalogeneerde koolwaterstoffen, en hun invloed op biologische systemen, de diversiteit van halorespirerende bacteriën die organohalides kunnen afbreken door middel van specifieke katalysatoren, de reductieve dehalogenases, waarvoor een ruim overzicht en twee modellen worden gepresenteerd die mogelijke reactiemechanismen beschrijven. Daarnaast worden gekarakteriseerde en potentiële transcriptionele regulatoren van halorespiratie en hun homologen geïntroduceerd, in het bijzonder het cAMP receptor eiwit (CRP) en de fumaraat nitraat reductie regulator (FNR) van

Escherichia coli. Speciale aandacht wordt gegeven aan aspecten van de structuur-functie relaties in CRP/FNR-type transcriptionele regulatoren en de complexe, multi-niveau controle van metabole processen in bacteriën. Tenslotte wordt het opkomende onderzoeksgebied van genomics van halorespirerende bacteriën gepresenteerd, gebaseerd op bioinformatica studies aan de genomen van vier halorespirerende bacteriën.

Hoofdstuk 2 beschrijft een nieuw lid van de CRP-FNR familie, CprK1, een transcriptionele regulator die een set van genen activeert die betrokken zijn bij halorespiratie in *D. hafniense*. Om de interactie te onderzoeken van CprK1 met drie van zijn voorspelde DNA-bindings sequenties die geïdentificeerd waren als 14-bp inverted repeats (dehaloboxes) in halorespiratie-geïnduceerde promoters, werden *in vitro* DNA-bindings assays alsook *in vivo* promoter-probe assays uitgevoerd. Er kon worden aangetoond dat CprK1 specifiek bindt aan dehaloboxes (consensus TTAAT----ATTAA) met hoge affiniteit in de aanwezigheid van 3-chloro-4-hydroxyphenylacetic acid (CHPA), en niet met het gedechloreerde derivaat HPA. Een gemuteerd CprK1 eiwit werd geconstrueerd door middel van een Val→Glu vervanging op een geconserveerde positie in de DNA-bindende herkennings α-helix, in een poging om de substraatspecificiteit te veranderen. De mutant verkreeg inderdaad een FNR-type DNA-bindings affiniteit, en herkende de sequentie TTGAT----ATCAA (FNR-box) in plaats van dehaloboxes, waarmee meer inzicht werd verkregen in de moleculaire basis van de specifieke eiwit-DNA interactie. CprK1 ondervond oxidatieve inactivering *in vitro*, wat waarschijnlijk veroorzaakt werd door de vorming van een intermoleculaire disulfide brug tussen Cys11 en Cys200. De mogelijkheid van redox-regulatie van CprK1 door een thiol-disulfide uitwisselingsreactie werd onderzocht door het construeren en karakteriseren van twee Cys→Ser mutant eiwitten. De verkregen resultaten suggereren dat ondanks dat de formatie van inactivatie door Cys11-Cys200 disulfide-bruggen, dit waarschijnlijk geen fysiologische rol speelt in de regulatie van CprK1 *in vivo*.

Ondanks het feit dat veel details van de transcriptie activatie van het CprK zijn bepaald door middel van eiwit-DNA bindingsstudies en promoter-probe analyses, blijft een aantal interessante vragen onbeantwoord. Hoe kan de regulator met nauwkeurigheid onderscheid maken tussen het gechloreerde effector molecuul CHPA en het niet-gechloreerde en biologisch inactieve derivaat HPA? Wat zijn de allostere effecten van CHPA binding die ertoe leiden dat CprK1 zijn specifieke DNA sequentie herkent en eraan bindt? Deze vragen komen aan bod in **Hoofdstuk 3**, waarin de 2.2 Å kristalstructuur van het geoxideerde CprK1 in complex met CHPA en de 2.8 Å kristalstructuur van het ligand-vrije, gereduceerde en sterk op CprK1 lijkende CprK van *D. dehalogenans* worden beschreven. Beide regulatoren zijn een homo-dimeer. In de monomeer structuren zijn drie domeinen te onderscheiden: een N-terminale β-barrel die de effector bindt, een lange dimerizatie α-helix en een C-terminaal DNA-bindings domein met een helix-turn-helix motief. De binding van CHPA veroorzaakt structurele veranderingen in de dimerizatie α-helix, doordat de residuen Tyr130 en Leu131 die zich in deze helix bevinden, contact maken met het chloor-atoom. Naast het ontbreken van deze interacties met het gedechloreerde derivaat HPA is er nog een factor van belang voor het allostere effect van CHPA op CprK1. Een geconserveerd lysine residue (Lys133 in CprK1) vormt een essentiële binding met de phenolate groep van CHPA.

Bij fysiologische pH is deze hydroxygroep in HPA geprotoneerd, waardoor een cruciale binding die betrokken is bij de repositionering van de N-terminale β-barrel niet kan worden gevormd (de zogenaamde "pK_a password" theorie). Behalve deze verschillen werd een onverwachte dimerizatie van de DNA-bindende α-helices geconstateerd in de CprK structuur zonder effector. Verondersteld wordt dat de conformatie veranderingen die door het ligand veroorzaakt worden (namelijk herpositionering van β strands 4 en 5) de dimerizatie van de C-terminale domeinen destabiliseert, waardoor nieuwe contacten tussen de domeinen kunnen optreden. Dit kan dan leiden tot een heroriëntatie van beide helix-turn-helix motieven die hierdoor de correcte DNA bindende orietatie kunnen aannemen.

In **Hoofdstuk 4** worden de structurele dynamica en de ligand-geïnduceerde conformatie veranderingen van CprK1 beschreven die met behulp van macromoleculaire massa spectrometrie, gedeeltelijke proteolyse en *in vitro* DNA-bindings studies zijn bestudeerd. Een kleine verzameling van potentiële effector moleculen waarvan bekend was dat ze gedehalogeneerd worden door *D. hafniense* DCB-2 of *D. dehalogenans*, is in deze experimenten gebruikt. Onder gereduceerde omstandigheden zorgden sterke effector moleculen (CHPA, 2,4,5-trichlorophenol; 2-Br-4-chlorophenol en 2,4-dichlorophenol) voor complexvorming met het dehalobox DNA. Phenol derivaten zonder gehalogeneerde substituenten in de *ortho* positie en een acetaat of halogeen-atoom in de *para* positie (bijvoorbeeld HPA; 2,3-dichlorophenol en 3,5-dichlorophenol) zorgden niet voor een allostere transitie van CprK1. Ook de geoxideerde vorm van CprK1 – waarbij een intermoleculaire zwavelbrug wordt gevormd tussen Cys11 en Cys200 – was niet in staat om DNA te binden. Daarom werd geconcludeerd dat zowel de reductie van het eiwit als de binding van een geschikt phenol derivaat (met halogeenatoom en/of acetaat substituent op de *ortho* en *para* positie) nodig zijn om de juiste conformatieverandering te bewerkstelligen die tot DNA binding leidt. De veranderingen in de structurele dynamica van CprK1 als gevolg van reductie, effector molecuul en DNA binding werden bestudeerd met behulp gedeeltelijke proteolyse gekoppeld aan massaspectrometrie. Over het algemeen was het eiwit flexibeler in gereduceerde vorm, omdat het eiwit onder gereduceerde condities twee maal zo snel werd afgebroken als onder geoxideerde omstandigheden. De binding van sterke effector moleculen induceerde veranderingen in de dynamica van de sensor module van het CprK1, terwijl het DNA-bindende domein gevoelig bleef voor protease digestie, onafhankelijk van de aan- of afwezigheid van effectors. De binding van het DNA is aangetoond door de bescherming van een 14-aminozuur peptide dat het geconserveerde V--SR motief bevatte, waarvan is aangetoond dat het betrokken is bij specifieke DNA herkenning (zoals beschreven in Hoofdstuk 2).

In de voorgaande hoofdstukken stond in het bijzonder de transcriptionele regulator CprK1 van *D. hafniense* centraal in het onderzoek. Echter, in de partiële genoom sequentie van *D. hafniense* zijn nog vier additionele genen aanwezig die voor CprK paralogen coderen, waarvan er drie clusteren met potentiële reductieve dehalogenase-coderende genen. In **Hoofdstuk 5** werd de rol van de verschillende CprK paralogen van *D. hafniense* bestudeerd door gebruik te maken van een *in vivo* promoter-probe assay in *E. coli* en *in vitro* DNA-bindings assays. De resultaten tonen aan dat twee regulatoren (CprK1 en CprK2) een overlappende effector-specificiteit hebben met een

voorkeur voor *ortho*-chloorfenol (CHPA), terwijl de effectoren van CprK4 *meta*-chloorfenolen (2,3-dichloorfenol en 3,5-dichloorfenol) zijn. De aanwezigheid van twee potentiële transposase-coderende genen in de nabijheid van de *cprK* genen duidt erop dat hun overrepresentatie waarschijnlijk het gevolg is van mobiele genetische elementen. De CprK-paralogen activeren transcriptie via promoters die een 14-bp inverted repeat (dehalobox) bevatten en die overeenkomst vertonen met de FNR-box. Er bestaat een sterke negatieve correlatie tussen de snelheid van transcriptionele regulatie en afwijkingen in de bindings-sequentie vergeleken met de optimale regulator-bindingssequentie. Een sterke halorespiratie-induceerbare promoter werd gevonden stroomopwaarts van een gen dat codeert voor een methyl-accepterend chemotaxis-eiwit, wat een eerste aanwijzing zou kunnen zijn voor chemotaxis voor gehalogeneerde aromatische verbindingen in een anaërobe bacterie.

Tenslotte beschrijft **Hoofdstuk 6** de eerste stap naar het uiteindelijke doel, nl. het ontrafelen van het reactiemechanisme van reductieve dehalogenases. Om de exacte rol te kunnen bestuderen van de cobalamine cofactor en de ijzer-zwavel clusters, en om de liganden die binden aan cobalamine (een algemeen B_{12}-bindingsmotief is niet aanwezig in het eiwit) te identificeren, zijn substantiële hoeveelheden van wild-type en mutant eiwitten noodzakelijk. Echter, productie van metallo-enzymen, en reductieve dehalogenases in het bijzonder, werd tot nog toe verhinderd door problemen met het verkrijgen van oplosbare recombinante eiwitten, welke vooral het resultaat zijn van de sterke zuurstofgevoeligheid van deze enzymen en de noodzaak van correcte cofactor inbouw in het eiwit. Daarom wordt een praktische aanpak beschreven voor de heterologe overproductie van twee cobalamine/ijzer-zwavel bevattende reductieve dehalogenases van *Desulfitobacterium* spp. door co-expressie van eiwitvouwings-katalysatoren die synthese van functionele eiwitten bevorderen. Wij konden aantonen dat gelijktijdige productie van de moleculaire chaperones GroEL/ES en een trigger-factor (TF), samen met geoptimaliseerde anaërobe groeiomstandigheden, resulteerde in overproductie van de reductieve dehalogenases, gedeeltelijk (10-15%) in de oplosbare celfractie van *E. coli*. Enzymatische activiteit van de recombinante enzymen was detecteerbaar, maar was significant lager dan de positieve controle (natuurlijke enzympreparatie), wat er waarschijnlijk op duidt dat een groot deel van de heteroloog geproduceerde enzymen geen correct geïncorporeerde co-factoren bevatten. Gebaseerd op recente resultaten, voorspellen wij dat vervanging van GroEL/ES en TF door de potentiële reductieve dehalogenase-specifieke chaperones (CprD, CprE) en trigger factor (CprT) uit *Desulfitobacterium* spp. wel zou kunnen resulteren in hogere productie van actiever CprA in *E. coli*; en daarmee in een protocol dat ook bevorderlijk zou kunnen zijn voor de overproductie van andere reductieve dehalogenases.

Samenvattend, draagt het beschreven onderzoek aan de transcriptionele regulatie en de katalytische basis van halorespiratie bij aan een beter inzicht (i) in de mogelijkheden voor bacteriële afbraak van met name antropogene milieuverontreinigingen, (ii) in de factoren die betrokken zijn bij de inductie van de bacteriële omzetting van dergelijke gehalogeneerde koolwaterstoffen, en (iii) in de voortdurende aanpassing van deze capaciteit in bacteriën.

Dit onderzoek draagt bij aan de ontwikkeling van een nieuw, in toenemende mate geaccepteerd concept in de milieubiotechnologie: in plaats van het ontwikkelen van verbeterde bioremediatie katalysatoren en "superorganismen" in de reageerbuis door middel van genetische manipulatie, proberen wetenschappers te begrijpen hoe bacteriën reageren op milieusignalen (zoals gehalogeneerde verontreinigingen), en hoe deze reactie geïntegreerd is in het globale regulatienetwerk, niet alleen in de individuele bacterie maar ook in de gehele microbiële gemeenschap[1]. Door inzicht te verkrijgen in de omstandigheden die van belang zijn voor de inductie van gewenste metabole activiteiten in de natuurlijke microbiële gemeenschap, zou het binnen afzienbare tijd mogelijk moeten zijn om bacteriële afbraak van milieuverontreinigingen te stimuleren door middel van eco-engineering.

1. Cases I, de Lorenzo V: Genetically modified organisms for the environment: stories of success and failure and what we have learned from them. Int. Microbiol. 8:213-22, 2005

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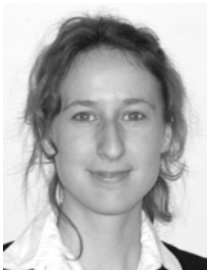
ABOUT THE AUTHOR



Krisztina Gábor was born on 1st of March 1978 in Budapest and grew up in the closeby town of Martonvásár (Hungary). She did her secondary studies in the József Attila Secondary Grammar School in Székesfehérvár between 1992-1996, while actively participating in the Herman László Music School and Conservatorium on violoncello class. Inspired by summer practical work in the Cell Biology group of dr. Beáta Barnabás at the Agricultural Research Institute in Martonvásár, in 1996 she begun her higher education at the Szent István University of Gödöllő, at the Faculty of Agricultural Sciences. In the fourth year of her studies she visited Wageningen University (The Netherlands) for a 6-months project on genetic map construction and QTL (quantitative trait loci) analysis of the ornamental plant *Alstroemeria* using amplified fragment length polymorphism markers.

In 2000 she continued her M.Sc. studies within the Environmental Sciences graduate program of Wageningen University. As part of this, she did a 7-months research project at the Laboratory of Microbiology under the supervision of prof. John van der Oost and dr. Ana López-Contreras on the genetic improvement of the cellulolytic properties of an industrially relevant *Clostridium acetobutylicum* strain. In 2001 she received her M.Sc. diploma from Wageningen University.

In October 2001 she started her doctoral research at the Laboratory of Microbiology, Wageningen University, focusing on the aspects of gene expression regulation and catalysis in the environmentally significant microbial process termed halorespiration. The results of the research – supervised by prof. Willem de Vos, prof. John van der Oost and dr. Hauke Smidt – are presented in this thesis.

Since September 2006, Krisztina Gábor works as a post-doc at the Technical University of Delft (The Netherlands) in the Department of Biotechnology. Her research is part of a larger project entitled Bio-Based Sustainable Industrial Chemistry (B-Basic) and aims at the discovery of industrially interesting enzymes from uncultivable micro-organisms by metagenomic tools.

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