Regulation of Anaerobic Dehalorespiration by the Transcriptional Activator CprK*

Desulmonomile, Desulfotobacterium, and Dehalobacter are anaerobic microbes that can derive energy from the reductive dehalogenation of chlorinated organic compounds, many of which are environmental pollutants. There is very little information about how anaerobic dehalorespiration is regulated. An open reading frame within the Desulfotobacterium dehalogenans chlorophenol reductase (cpr) gene cluster (cprK) was proposed to be a transcriptional regulatory protein (Smidt, H., van Nol reductase (CprK) was suggested to be a transcriptional regulatory protein (Smidt, H., van

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The regulation of anaerobic dehalorespiration is significant barrier to metabolizing these compounds, and anaerobic bacteria are considered more efficient than aerobes in removing the halogen atoms from the polychlorinated biphenyls (2). Several reviews on the microbiology and enzymology of dehalogenation are available (2–6).

Several classes of anaerobic bacteria can use different halogen-containing compounds as electron acceptors and, thus, harvest energy from the reductive dehalogenation reaction (Reaction 1) in a process called dehalorespiration. With pyruvate as the electron donor, Desulfotobacterium dehalogenans generates 1 mol of ATP/mole of chloride removed through formation of a protonotive force (7). In other organisms, less than 1 mol of ATP is gained per chloride liberated (8, 9). Approximately 20 strains of anaerobic organisms capable of dehalorespiration have been isolated, including Desulmonomile, Desulfotobacterium, and Dehalobacter. The low G + C, Gram-positive bacterium D. dehalogenans, from which the protein studied here originates, can utilize hydroxylated polychlorinated biphenyls (10), and the Desulfotobacterium chlororespirans dehalogenase can use hydroxy-polychlorinated biphenyls as substrates (11). Multiple gene clusters encoding reductive dehalogenases have been detected in the genomes of dehalorespiring microbes, including 17 potential dehalogenases in Dehalococcoides ethenogenes (12). Some of the proteins involved in dehalorespiration are encoded in the cpr (chlorophenol reduction) gene cluster, which includes the reductive dehalogenase (cprA), its membrane anchor (cprB), several proposed chaperones (cprT, -Z, -E, and -D), and two putative regulatory proteins (cprC and cprK) that are organized into four transcriptional units encoding cprT, cprBA, cprZE, and cprBACD (13). Another transcript cprTZE was also detected.

\[ \text{R-Cl} + 2e^- + 2H^+ \rightarrow \text{RH} + \text{HCl} \]

**REACTION 1**

It is important to understand how dehalorespiring microbes regulate expression of the cpr genes. CprK was suggested to be a transcription regulatory protein based on its sequence similarity to fumarate nitrate regulatory protein (FNR)† and FixK (13), which are global activators of gene expression that control transcription initiation in response to oxygen limitation (14). CprK also exhibits similarity to the cAMP receptor protein (CRP), which controls catabolite repression by activating transcription at over 100 different promoters in Escherichia coli (15). CRP and FNR regulate transcription by recognizing and binding to a 22-base pair binding sequence upstream of target promoters, which is known as the “FNR box” (16, 17). The

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The abbreviations used are: FNR, fumarate nitrate regulatory protein; EMSA, electrophoretic mobility shift assay; CPA, 3-chloro-4-hydroxyphenylacetate; HPA, 4-hydroxyphenylacetate; CPA, 3-chlorophenylacetate; CRP, cyclic AMP receptor protein; NTA, nitritolatriacetic acid; ITC, isothermal titration calorimetry.
upstream promoter region of the four cpr transcripts contains an "FNR-like box" that overlaps the “minus 35” region (13). These similarities suggest CprK is a transcriptional regulator of respiration on chloroaromatic compounds. However, unlike FNR, CprK lacks any obvious motifs for FeS clusters (13), which makes it similar to FixK. On the other hand, CprK does show an unusually high content of five cysteine residues, among which is a conserved internal cysteine residue (Cys105). In the E. coli FNR protein, the corresponding Cys122 has been shown to be essential for iron binding, disulfide bond formation, and covalent modification (18). Here we have used in vitro and in vivo approaches to demonstrate that CprK is indeed a transcriptional activator, demonstrated the specificity of its effector domain, revealed redox activation of CprK, and addressed the role of the “FNR-like box.”

EXPERIMENTAL PROCEDURES

**Bacterial Strains and Growth Conditions—** E. coli strains were used as Top10 and JM109 (Invitrogen) for construction and maintenance of plasmids, Rosetta (DE3) (Novagen EMD Biosciences, Inc., Madison, WI) for expression of CprK and CprB, and E. coli BL21 (DE3)-CprK and E. coli BL21 (DE3)-CprB (pGEM) for gel mobility shift assays. Vectors used include phagemid pGEM-Sf (Promega, Madison, WI), pQE60 (Qiagen Inc., Chatsworth, CA), plasmid pRS551, which was provided by Professor Jorge Escalante-Semerena (University of Wisconsin, Madison, WI). The chemical reagents used here were purchased from Sigma, and the N2 (99.998%) was from Linde (Lincoln, NE). Wisconsin, Madison, WI). The chemical reagents used here were purchased from Sigma, and the N2 (99.998%) was from Linde (Lincoln, NE).

**E. coli** cells were grown in Terrific Broth (1.2% tryptone, 2.4% yeast extract, and 0.4% glycerol supplemented with 1 x TB salts (2.3% KH2PO4, 12.5% K2HPO4)), except for LMG194, which was grown in RM medium + glutamate (1% K2HPO4, 0.3% KH2PO4, 0.5 mM NaCl, 0.1% NiCl2) at 37 °C. For anaerobic growth of *E. coli*, we used LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 40 mM sodium fumarate, 1% glucose, 20 mM ferrous ammonium sulfate, and 100 μM sodium sulfite. Antibiotics were used at final concentrations of ampicillin, 25 μg/ml for ampicillin, 25 μg/ml for kanamycin, and 34 μg/ml for chloramphenicol.

**Cloning and Overexpression of CprK—** General DNA isolation and manipulation were performed using standard techniques (19). Plasmid DNA was purified with a QiAprep spin miniprep kit (Qiagen). DNA fragments were purified from agarose gels using a QIAquick gel extraction kit (Qiagen). To obtain a PCR template, a 0.5-ml aliquot of an *E. coli* cell extract was loaded on a column of nickel-nitrotriacetic acid (Ni2+-NTA) (Qiagen) followed by washing with buffer containing 20 mM imidazole and eluting with a gradient from 20 to 250 mM imidazole according to the manufacturer's instructions. The purification steps were performed in the glove box under anaerobic conditions at 12 °C or in a cold room under aerobic conditions at 4 °C.

**Determination of Protein Concentration and Purity—** Protein concentrations were determined using the Rose Bengal method (20). The purity of CprK protein preparations was estimated by SDS-PAGE (21). The N-terminal protein sequence was determined using an automated sequencer (22) by the Biotechnology Proteonomics Research Core Facility at the University of Nebraska, Lincoln. The protein mass was determined by electrospray ionization mass spectrometry with a QStar XL (quadrupole/time and flight) by the Mass Spectrometry Core Facility at the University of Nebraska, Lincoln.

**DNA Sequencing—** The DNA sequences of all PCR-generated DNA fragments were confirmed by automated sequencing of both strands by the Genetics Core Research Facility, University of Nebraska, Lincoln (23) with a Beckman/Coulter CEQ2000XL eight-capillary DNA sequencer using dye terminator chemistry.

**Gal Retardation or Electrophoretic Mobility Shift Assay (EMA)—** A 193-bp fragment containing the CprB promoter region was amplified by polymerase chain reaction using the primers PcrB1 (5'-GATATCGGG-AATAGTCCGACCCGCAACGG-3') and PcrB2 (5'-GATATCTCCACCCGAAAGGCG-3') (where the introduced EcoRV site is shown in boldface type). The PCR product was inserted into the EcoRV site within the multiple cloning site of the pGEM-Sf (+) vector, resulting in pGEM-PcprB. The 193-bp EcoRV fragment was excised from pGEM-PcprB and 5'-end-labeled by γ-32P-ATP (3000 Ci/mmol; American Biosciences) and used in a gel retardation assay (EMA). Smaller DNA fragments were generated using the procedure described above with the following primers: PcrB3 (5'-GATATCGGCATCTTCTCATCCTTCT-3') and PcrB2 from above for a 140-bp fragment containing the CprB promoter region; PcrB4 (5'-GATATCGGACATCACTGTTAGTTGTC) and PcrB3 for a 90-bp fragment; PcrB5 (5'-GATATCAAGAAATTCTGAGATTAATATTAC) and PcrB2 for a 100-bp fragment; and PcrB4 and PcrB5 for a 50-bp fragment. All of the above primers were synthesized by Integrated DNA Technologies (Coralville, IA). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.25 mM MgCl2, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM dithiothreitol. Samples were degassed by vacuum aspiration for 5 min prior to loading, and all titrations were performed at 25 °C. Autoradiograms were obtained using a BioRad Gel Doc 2000 apparatus.

**Isothermal Titration Calorimetry Experiments—** For ITC experiments, purified and refolded CprK was dialyzed against a buffer containing 150 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10 mM dithiothreitol. The concentration of CprK was determined by absorbance at 280 nm, and the thermal power of 60 injections of 3 μl was recorded every 240 s. Thermogravity analysis was performed using the Origin 7.0 software supplied with the instrument.

**Construction of Strains for lacZ Reporter Assays—** To measure its activity under tight control of expression, cprk was cloned into the pBAD-Myc-HisA expression vector (Invitrogen), generating pBAD/Myc-HisA::cprk. A fusion between the promoter region of cprk and lacZ was generated by cloning the promoter region in the pBAD-Myc-HisA::cprk. This mixture was incubated at room temperature for 10 min, 32P-labeled DNA fragment PcrB was added, and the mixture was incubated for 10 min at room temperature. The mixtures were separated by electrophoresis under native conditions (10% polyacrylamide gel) for 1 h with thiglyglycolic acid to remove any oxidants remaining in the gel) buffer = 1 x Tris-glycine-EDTA (50 mM Tris-HCl, 380 mM glycine, and 2 mM EDTA, pH 8.2) and run at 140-V constant voltage, followed by drying and autoradiography.

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**Site-directed Mutagenesis of CprK—** The conserved Cys105 residue
Transcriptional Regulator of Dehalorespiration

Cloning and Overexpression and Purification of CprK—To study the mechanism by which expression of the dehalorespiration genes is regulated, we overexpressed and purified the putative transcriptional regulatory protein CprK. The cprK gene from *D. dehalogenans* was PCR-amplified and cloned in frame with a C-terminal His<sub>6</sub> tag in the pQE60 vector, transformed into *E. coli* Rosetta (DE3) cells, and grown aerobically in Terrific Broth medium. Recombinant CprK was then purified with an Ni<sup>2+</sup>-NTA column to more than 98% homogeneity (Fig. 1).

Purified CprK exhibits the expected molecular mass of 28 kDa based on SDS-PAGE (Fig. 1) and the expected N-terminal sequence for the first 10 amino acids. The molecular mass determined by mass spectroscopy, 27,947.1 ± 2.7 Da, was also in agreement with the expected value. Based on gel filtration experiments, the protein is predominantly in a homodimeric state, but significant amounts of the monomer (~30%) are observed, indicating a dynamic equilibrium between the monomeric and dimeric forms (data not shown). UV-visible spectroscopic studies indicate that CprK does not contain a chromophore with an absorption spectrum in the 300–640-nm region. Based on 30-element inductively coupled plasma-emission spectrometry (Chemical Analysis Laboratory, University of Georgia, Athens, GA) (26), which includes all metals known to be present in enzymes, the only metal found was zinc, and this was present in only substoichiometric amounts (0.2–0.3 per mol). However, as described below, zinc does not appear to be required for activity.

CprK- and CHPA-dependent DNA Binding—To test whether CprK binds the “FNR-like” box in the promoter region of the cpr genes, as predicted by nucleotide sequence analysis (13), EMSAs were performed with CprK and a 193-bp DNA fragment containing the promoter region of the *cprB* gene from position –156 to +37. Compared with free DNA, the position of the DNA band is significantly retarded when incubated with CprK and the dehalorespiration substrate CHPA (Fig. 2). In the absence of CHPA or CprK, there is no gel shift. Initially, the EMSAs were unreliable, with only freshly purified protein appearing to be active. The EMSA results became highly reproducible after it was recognized that the aerobically purified protein is inactive and that aerobically purified protein regains full activity in the presence of 1 mM isopropyl-thio-β-D-galactoside. The purity of CprK after elution from the Ni<sup>2+</sup>-NTA column was analyzed on 12% SDS-PAGE stained with Coomassie Brilliant Blue. Lane 1, a low range molecular marker; Lane 2, Ni<sup>2+</sup>-NTA column-purified CprK was mutated to alanine using the QuikChange<sup>®</sup> site-directed mutagenesis protocol from Stratagene. The pBAD/Myc/HisA::cprK plasmid was the template for PCRs using the following primers from Integrated DNA Technologies: 5′-TATGGAAACAGAACAGCCCTGTTT-TCCGAAAGATG-3′ (forward) and 5′-ACATTCTTCGGAAAACCAG-GCCTCTTCTGCTTGTCAT-3′ (reverse).

Quantification of Soluble CprK in the in Vivo lacZ Reporter Assays—Quantification of CprK in cultures used in the β-galactosidase assays was accomplished by Western blotting and densitometric analysis. Aliquots equal to 1 ml of a culture in exponential growth phase with an absorbance at 600 nm of 1.0 were centrifuged, and the pellet was resuspended in SDS denaturing buffer and loaded on 15% SDS-polyacrylamide gels along with known quantities of purified Histagged CprK. After electrophoresis, protein was electroblotted onto nitrocellulose and reacted with rabbit antibody against CprK (obtained from Cocalico Biologicals Inc., Reamstown, PA) that had been purified using an Actigel ALD Superflow affinity column (Sterogene Bioseparations Inc., Carlsbad, CA) that had been reacted with CprK. Secondary antibodies were goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). CprK was quantified using a Bio-Rad universal hood and Quantity One software. The amount of CprK in each sample was measured by comparing the densitometry-determined staining intensity of the CprK band from the culture with that of the standard protein. To calculate the number of molecules/cell, we assumed that a culture with an absorbance of 600 nm of 1.0 contained 10<sup>9</sup> cells/ml. To assess the amount of soluble versus insoluble CprK, a separate sample of the exponentially growing cultures was centrifuged, resuspended in 1 ml of lysis buffer containing 0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3% KH<sub>2</sub>PO<sub>4</sub>, 0.1% NH<sub>4</sub>Cl, 0.05% NaCl, and 1 mM MgCl<sub>2</sub> (pH 7.4), and lysed by incubation with 1 mg/ml lysozyme for 30 min on ice and then sonication (1.5-min total sonication time at 40 watts using 1-s pulses with 5-s intervals) and centrifugation at 16,000 × g for 20 min. After removing the supernatant, the pellet fraction was washed with lysis buffer and centrifuged again at 16,000 × g for 20 min, and the wash and supernatant were combined. The pellet fraction was solubilized in SDS denaturing buffer, and the two fractions (supernatant and solubilized pellet) were loaded onto 15% SDS gels. Western blotting and densitometric analysis were performed as described above to determine the percentage of soluble and insoluble CprK.

DNase I Footprinting—DNase I footprinting assays were performed by digesting end-labeled plasmid pGEM::PcprB containing the *cprB* promoter region with either NcoI or SpeI and end labeling at the 5′ ends by the T4 polynucleotide kinase enzyme reaction with [γ<sup>32</sup>P]ATP, followed by digestion with the other restriction enzyme (SpeI or NcoI). This protocol generates fragments labeled at only one end. After isolation and purification, the labeled fragment was incubated with CprK as described for the EMSA and digested with RNase-free DNase I as described by the manufacturer’s guidelines (Promega). After quenching the reactions with EGTA, the DNA was precipitated, re-suspended, heat-denatured, and separated on a 7 M urea–8% polyacrylamide sequencing gel. The DNA sequencing ladder was generated by the dideoxy chain termination method (25) with a T7 Sequencing Kit (U.S. Biochemical Corp.), loaded beside the corresponding footprinting reactions.

RESULTS

**Fig. 2. Effector specificity of CprK.** This EMSA experiment was performed in the presence of CHPA or CHPA analogs. Except for lane 1, all reactions contain 10 μM CprK and 500 μM potential effector, except for CHPA, which was used at a 20 μM final concentration. Lane 1, no substrates; lane 2, CHPA; lane 3, CFA; lane 4, HPA; lane 5, CAMP; lane 6, 3-chloro-4-hydroxybenzoate; lane 7, 3-chlorobenzoate; lane 8, 3,5-dichloro-4-hydroxybenzoate; lane 9, 2,4-dichlorobenzoate.
activity upon incubation with 2 mM dithiothreitol, which is a reducing agent. Further studies of the redox regulation of CprK are described below.

To determine the specificity of CprK for its effector, several potential analogs of CHPA, which is a terminal electron acceptor for *D. dehalogenans*, were compared in EMSAs. Although CprK exhibits significant sequence similarity to CRP, including the region within the effector (cAMP) binding domain, cAMP does not promote a gel shift (Fig. 2), and elevated levels of cAMP do not inhibit the gel shift induced by CHPA (data not shown), indicating that cAMP does not bind to the effector domain of CprK. At 0.5 mM concentrations, hydroxyphenylacetate, which is structurally related to CHPA but lacks the ortho-chlorine group, chlorophenylacetate (which lacks the hydroxyl group), and pentachlorophenol lacking the carboxyl group (Fig. 2) do not induce DNA binding by CprK. Chlorohydroxybenzoate (0.5 mM) appears to act as a weak effector relative to CHPA.

The intensity of the gel-shifted band depends on the concentration of CprK (Fig. 3) and CHPA (Fig. 4). The $K_d$ for CprK of 190 nM indicates a strong interaction with DNA. At very high concentrations of CprK, a "supershift" is observed (data not shown), which presumably originates from a CprK-DNA complex involving oligomeric states of CprK. By varying the concentration of CHPA, a dissociation constant of 0.40 μM can be calculated (Fig. 4), which indicates a very strong protein-effector interaction.

Since the EMSA assays are performed in the presence of DNA, the $K_d$ that is determined reflects the binding of effector to form a ternary (CHPA-CprK-DNA) complex. To obtain more quantitative information about formation of the binary CHPA-CprK complex, isothermal titration calorimetry experiments were performed (Fig. 5). CHPA binding to CprK is exothermic.

The apparent $K_d$ was 3.5 ± 0.4 μM with a stoichiometry ranging from 0.55 to 0.84 CHPA per CprK monomer, indicating that the His-tagged protein is 55–80% active, at least in terms of effector binding. Based on comparing the $K_d$ values from the ITC (binary complex) with the EMSA (ternary complex) results...
Complement the transcriptional activity of CprK of DNA binding activity. In one experiment, EDTA was reacted with CprK for 30 min before the EMSA experiment was begun.

The His tag sometimes inactivates proteins, and, when CprK is provided by the tightly regulated pBAD plasmid, which is induced by arabinose, and the P(cprB)-lacZ fusion is present on another plasmid. In this construct, CprK is provided by the tightly regulated pBAD plasmid, which is induced by arabinose, and the P(cprB)-lacZ fusion is present on another plasmid. β-Galactosidase activity (Miller units) 74.8 ± 4.9 77.1 ± 3.3 1371 ± 52 3656 ± 93
Specific activity 8.4 ± 0.8 11.3 ± 0.7 68.7 ± 4.1 287 ± 26

Average values are calculated from three data points for total protein determination and four separate reactions for the β-galactosidase assay. Error limits equal one S.D.

Comparison of the in vivo activities of C-terminal His tag and untagged CprK

The His tag sometimes inactivates proteins, and, when CprK is expressed with an N-terminal His-tag, it is completely inactive both in vivo and in vitro (data not shown). To quantitatively assess the effect of the C-terminal His tag on the structure and function of CprK, both the in vivo activity in the lacZ reporter assay and intracellular solubility of the His-tagged and untagged form of CprK were measured and compared. The His tag appears to slightly decrease the solubility of CprK; however, both the tagged and untagged proteins are nearly 100% soluble (Table I). By comparing Western analysis and the lacZ reporter assay results, the specific activities in terms of Miller units of β-galactosidase produced per pmol of soluble CprK were calculated. By comparing the specific activities, which represent the in vivo DNA-binding affinities, for the His-tagged and untagged forms of CprK in the absence and presence of 50 μM CHPA (Table I), it appears that removal of the His tag from CprK results in a 1.3-fold increase in DNA binding affinity in the absence of CHPA and a 4.2-fold increase in DNA binding affinity in the presence of 50 μM CHPA (Fig. 7 and Table I).

Redox Regulation of CprK—Treating CprK with diamide, which is commonly used to oxidize thiols to the disulfide state, eliminates DNA binding (Fig. 8). This is consistent with the oxygen sensitivity of the protein described above. Reaction of the diamide-treated enzyme with dithiothreitol completely reverses the inactivation. Treatment with EDTA also affects the activity of CprK, suggesting that metal ions could influence the activity of CprK; however, CprK contains less than stoichiometric amounts of metal ions (we have not observed zinc contents higher than 0.4 g atom/mol), and the addition of Zn(II), Co(III), and Fe(II) does not affect activity in the EMSA experiments (data not shown). Subjecting the purified protein to a typical FeS reconstitution protocol (27)
did not alter the UV-visible spectrum of CprK, which shows
typical absorption bands for a protein, with a peak at 280 nm
and no bands in the 350–420-nm region (data not shown).
These results indicate that CprK, unlike FNR, lacks the
ability to form an FeS cluster and suggests that its activity is
regulated through the reversible oxidation/reduction of a cys-
teine residue(s), perhaps involving thiol/disulfide bond
equilibria.
CprK contains a cysteine residue (Cys105) that aligns with
Cys122 of *E. coli* FNR, which is essential for iron binding,
disulfide bond formation, and covalent modification (18). When
the *in vivo* activity of the C105A mutant of CprK was compared
with that of the wild-type protein, there was no significant
difference in their *in vivo* activities. This result clearly indi-
cates that the conserved residue Cys105 is not essential for
binding CHPA or DNA.

**Identification of CprK-specific Binding Sequence**—The same
193-bp promoter region used in EMSA experiments was sub-
jected to DNase I protection experiments (Fig. 9A). A specific
pattern of protection was detected only when the labeled pro-
mer fragment was incubated with both CprK and the effector
(Fig. 9A). There was no protection in the absence of CHPA. The
CprK-protected site covers 14 bases between positions −35 and
−48, which is a sequence containing dyad symmetry (Fig. 9A)
with similarity to the consensus sequence motifs recognized by
CRP and by FNR in *E. coli* (Fig. 9B).

Further support for this binding sequence was obtained by
performing EMSAs with DNA fragments of varying length
(190, 140, 90, 100, and 50 bp) to determine the minimum DNA
sequence required by CprK for binding (Fig. 9C). The 50-bp
DNA fragment centered at the FNR-like binding site is suffi-
cient for CHPA-dependent high affinity CprK interaction with
the *cpr* promoter region.

**DISCUSSION**

The work described here builds primarily on studies by
Smidt *et al.* (13), who identified and characterized the *cpr* gene
cluster encoding proteins involved in the reductive removal of
the halogen group from CHPA and related chlorinated aromat-
ces. They showed that the *D. dehalogenans* *cprK* gene is part of
the *cprKZE* transcript and is expressed constitutively at low
levels. Furthermore, CprK was suggested to be a transcrip-
A transcriptional regulatory protein based on its sequence similarity to FNR and FixK, which are global regulators of anaerobic metabolism. They also identified a palindromic sequence motif (TTAAT-N4-ACTAA) upstream of three of the four transcripts in the cpr gene cluster that is similar to the “FNR box” (TTGAT-N4-ATCAA) (14), to which FNR binds.
Here we describe experimental support for the hypothesis that CprK is a transcriptional regulatory protein and provide information about how this protein interacts with its effector and with DNA to regulate expression of genes involved in the dehalorination of chlorinated aromatics. Fig. 10 depicts a testable working model for how CprK interacts with RNA polymerase and the promoter region containing the FNR-like box to promote transcriptional activation of the cpr gene cluster.

The *D. dehalogenans* CprK was actively overexpressed in *E. coli* with a His tag and purified to homogeneity using nickel affinity chromatography. The protein is predominantly a homodimer of two 28-kDa subunits. The C-terminal His-tagged and untagged proteins exhibit similar solubilities in the cell, and the N-terminal His tag appears to decrease the activity of CprK by ~4-fold based on the *in vivo* DNA binding studies. However, the His-tagged protein exhibits strong interactions with CHPA with a stoichiometry of 0.55 and 0.84 mol of CHPA/CprK monomer. Although the C-terminal His tag does exert a mild effect on activity, because of the ease of purification and the relatively higher degree of stability (not shown), we performed our *in vitro* experiments with the His-tagged protein. Whereas the C-terminally tagged protein is active, the protein containing an N-terminal His tag is completely inactive *in vivo* and *in vitro* (data not shown).

As shown in Fig. 2, CprK, like the dehalogenase (CprA) (11), is highly specific and only binds DNA in the presence of its effector (e.g. CHPA). It binds CHPA with a *Kd* value of 0.4 \( \mu \text{M} \); however, CprK does not respond to CFA or HPA at concentrations at least 100-fold higher than the *Kd* for CHPA, indicating that the cis-arrangement of hydroxyl and chlorine groups is optimal for activity. A similar specificity was observed for dehalorespiratory growth of *D. dehalogenans* cultures (28) and reductive dehalogenation by the purified dehalogenases (CprAs) from this organism (29) and from *D. chlororespirans* (11). Interestingly, substrate recognition by the transcriptional activator CprK is about 10-fold more sensitive than that of the dehalogenases, which exhibit *Km* values in the 10–20 \( \mu \text{M} \) range (11, 29). Thus, it appears that CprK is indeed the transcriptional activator controlling expression of the CHPA-regulated cpr gene cluster. Once a particular environment is depleted of the chlorinated hydroxylated aromatic compound, the cpr operon would be repressed (or deactivated), in agreement with the conclusion that dehalorespiring microbes do not further metabolize these compounds. After the chloro group is removed, other microbes are required for further metabolism of these aromatics.

Based on *in vitro* EMSA and footprinting assays and *in vivo* promoter-lac fusion experiments, CprK binds to a 22-bp sequence upstream of the “−35 region” that strongly resembles the FNR box, which is the binding site for the anaerobic regulator, FNR. Furthermore, CprK contains an N-terminal region that is structurally homologous to the effector domain and C-terminal region with homology to the DNA-binding region of both CRP and FNR, indicating that CprK is a new member of the CRP and FNR family of transcriptional activators. As Guest and co-workers (30) point out, the structural fold in the effector domain is versatile enough to accommodate a FeS cluster in FNR, a nucleotide binding fold in CRP, a heme in CooA (a CO-dependent transcriptional activator), and, as shown here, a chloroaromatic binding site for CprK. The helix-turn-helix motif in these proteins is also conserved and functions in binding to the “FNR-like box” region of Class II promoters, which contain a 22-base sequence centered approximately at position −41 overlapping the −35 element. However, it is essential that CprK bind exclusively to its own cognate DNA binding region and not that for FNR or other activators, a specificity that is probably conferred by slight changes in the FNR-like box sequence and in the amino acid residues in the DNA binding region of CprK. In this class of proteins, interaction with RNA polymerase in activating regions 1, 2, and 3 confers transcriptional activation (17, 30). As demonstrated by the promoter-lac fusions, CprK is a highly efficient transcriptional activator in the *E. coli* background, strongly suggesting that these same activating regions that are present in FNR and CRP are shared with CprK, although further studies are required to determine whether CprK is most like FNR or CRP, which differ in the roles of activating regions 1 and 2 (31). Although another putative regulatory protein (CprC) is present in the cpr gene cluster, efficient CHPA-dependent transcriptional activation of genes containing the “FNR-like” box can be observed in an *E. coli* background devoid of CprC. Further studies will be required to assess whether CprC provides another layer of regulation, perhaps by interacting with CprK and/or perhaps controlling its redox state.

Incubation of CprK under aerobic conditions leads to inactivation, which is reversed by the addition of the reducing agent, dithiothreitol. When it is purified and maintained under anaerobic conditions, CprK maintains its CHPA-dependent DNA binding activity for at least 3 weeks. Diamide treatment inactivates CprK in a dithiothreitol-reversible manner, suggesting that it is subject to redox regulation. Our working hypothesis is that the protein is controlled by the redox state of one or more of its five cysteine residues, which could be involved in disulfide/dithiolate interconversion, as suggested in Fig. 10. A single cysteine could be involved if the protein forms an intermolecular SS bridge or is regulated by reversible formation of the cysteine sulfenic acid or sulfenic acid (32, 33). The only conserved cysteine residue is Cys^{105}, and mutation of this residue
has no apparent effect on activity. However, it is still possible that Cys\(^{105}\) is involved in redox control of activity and that, in the absence of oxidative stress, the cytoplasm of \(E.\ coli\) could be sufficiently reducing to maintain the wild-type protein in a fully reduced state. If reversible formation of an SS bond is the only function of Cys\(^{105}\), the C105A mutation could mimic the fully reduced form of CprK, since it would be unable to form a disulfide bond. Experiments are under way to quantitatively determine the effect of the mutation on the in vitro and in vivo activities of CprK under oxidative stress conditions.

Since desulfotobacteria are anaerobes, down-regulation of the cpr gene cluster may be a way to protect the organism from the potentially harmful effects of oxygen reacting with the dehalogenase, a \(B_{12}/FeS\) enzyme. Further studies are required to understand the origin of the oxygen sensitivity. A number of cellular processes in prokaryotes and eukaryotes are regulated by the redox state of specific cysteine residues, including, among many others, transcriptional regulation by OxyR (34), glutathione biosynthesis (35), progression through the cell cycle (36), iron metabolism by FurS (37), DNA binding by NF-kB (38), and thioredoxin/thioredoxin reductase-dependent reactions (39, 40).

Conclusions—CprK is a new member of the FNR/CRP family of transcriptional activators. Its effector domain is highly specific for chloroaromatics with a cis-arrangement of hydroxy and chlorine groups. Binding of CHPA, the preferred substrate for \(D.\ dehalogenans\), triggers interaction with a 14-bp DNA binding motif of dyad symmetry in the promoter region of genes involved in dehalorespiration. This sequence bears strong similarity to the FNR-box that is the target for FNR. Rich in cysteine residues but apparently lacking metals, CprK is inactivated by diamide and reactivated by dithiothreitol, indicating that one or more of its five cysteine residues undergo disulfide/dithiol interconversion and suggesting that CprK is subject to redox regulation. This is the first characterization of a transcriptional regulator of dehalorespiration.

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