Desulfitobacterium dehalogenans can use chlorinated aromatics including polychlorinated biphenyls as electron acceptors in a process called dehalorespiration. Expression of the cpr gene cluster involved in this process is regulated by CprK, which is a member of the CRP/FNR (cAMP-binding protein/fumarate nitrate reduction regulatory protein) family of helix-turn-helix transcriptional regulators. High affinity interaction of the chlorinated aromatic compound with the effector domain of CprK triggers binding of CprK to an upstream target DNA sequence, which leads to transcriptional activation of the cpr gene cluster. When incubated with oxygen or diamide, CprK undergoes inactivation; subsequent treatment with dithiothreitol restores activity. Using mass spectrometry, this study identifies two classes of redox-active thiol groups that form disulfide bonds upon oxidation. Under oxidative conditions, Cys105, which is conserved in FNR and most other CprK homologs, forms an intramolecular disulfide bond with Cys111, whereas an intermolecular disulfide bond is formed between Cys11 and Cys200. SDS-PAGE and site-directed mutagenesis experiments indicate that the Cys11/Cys200 disulfide bond links two CprK subunits in an inactive dimer. Isothermal calorimetry and intrinsic fluorescence quenching studies show that oxidation does not change the affinity of CprK for the effector. Therefore, reversible redox inactivation is manifested at the level of DNA binding. Our studies reveal a strategy for limiting expression of a redox-sensitive pathway by using a thiol-based redox switch in the transcription factor.

Desulfitobacterium dehalogenans is a Gram-positive bacterium that can recognize and use chlorinated aromatics, like polychlorinated biphenyls and chlorohydroxyphenyl acetate (CHPA), as electron acceptors (1, 2) in an energy yielding process called dehalorespiration (3, 4). Dehalorespiration couples the two-electron reduction of the chlorinated aromatic compound and elimination of the chloride group (dehalogenation) to oxidation of membrane-associated electron carriers and to proton translocation, ultimately resulting in ATP generation (3, 5–7).

Polychlorinated biphenyls and other haloaromatics are xenobiotics that are produced by industry and also are biologically generated (8). Exploiting the ability of dehalorespiring microbes to initiate degradation by removal of the halogen group is a potential strategy for bioremediation of these toxic compounds.

Induction of dehalorespiration activity occurs when desulfitobacteria are exposed to a chlorinated aromatic compound that can serve as a dehalogenation substrate (3). Induction is mediated through the transcriptional regulatory protein, CprK. Binding the chloroaromatic compound as an effector molecule triggers a conformational change in CprK, which promotes productive interaction with a nearby palindromic DNA sequence called the “FNR-like box” located upstream of four proximal transcriptional units in the cpr regulon (9). The cpr regulon contains eight genes, cprT, cprK, cprZ, cprE, cprB, cprA, cprC, and cprD, that encode the dehalogenase (CprA), CprK, and a number of proteins assigned as chaperones and other components of the dehalorespiration process.

The role of CprK as a transcriptional activator of the cpr dehalorespiration genes and its interaction with the FNR-like box had been predicted earlier by Smidt et al. (10) when the cpr regulon was first described. This proposal was confirmed by in vivo and in vitro DNA binding studies, which also demonstrated that CprK only functions under reducing conditions (9). Exposure to oxygen or incubation with a reagent (diamide) that oxidizes dithiols to their disulfide form inhibits binding of CprK to the FNR-like box sequence, and treating the oxidized protein with dithiothreitol reverses the oxidative inactivation (9). The cellular rationale for redox regulation of CprK activity may relate to the oxygen sensitivity of the dehalogenase CprA, which is a metalloenzyme containing two iron-sulfur clusters and a modified form of vitamin B12 (11). Because it would be wasteful to produce CprA under oxidative conditions, it would be advantageous to control cpr transcription as a function of the redox state of the cell.

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4 The abbreviations used are: CHPA, 3-chloro-4-hydroxyphenylacetate; EMSA, electrophoretic mobility shift assay; ITC, isothermal titration calorimetry; DTT, dithiothreitol; IAM, iodoacetamide; VP, vinyl pyridine; MS, mass spectrometry.
In this study, the mechanism of redox regulation of CprK activity was examined. Oxidation of CprK was shown by internal quenching of fluorescence experiments and by isothermal calorimetry measurements to have no affect on the affinity of CprK for effector, strongly suggesting that oxidative inactivation is exhibited at the level of DNA binding. By mass spectrometry, nonreducing SDS-PAGE, and site-directed mutagenesis studies, the reversible redox inactivation was localized at one intermolecular and one intramolecular disulfide bond.

MATERIALS AND METHODS

Cloning, Overexpression, and Purification of CprK—DNA isolation and manipulation were performed using standard techniques (12). 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). Construction of the overexpression plasmid and purification of CprK were described previously (9).

Site-directed Mutagenesis of CprK—Cys$^{11}$ and Cys$^{200}$ were substituted with serine residues using the QuikChange site-directed mutagenesis protocol from Stratagene (La Jolla, CA). The pQE60::cprK plasmid was the template for PCRs using primers from Integrated DNA Technologies.

DNA Sequencing—The DNA sequences of all PCR-generated DNA fragments were confirmed by automated sequencing of both strands by the Genomics Core Research Facility (University of Nebraska-Lincoln) with a Beckman/Coulter CEQ2000XL 8-capillary DNA sequencer using dye terminator chemistry.

Alkylation of Cysteine Residues—Reduced (with 10 mM dithiothreitol (DTT)) and oxidized (lacking DTT) CprK samples at a concentration of 2 mg/ml in 0.5 ml of Buffer A (50 mM Tris-HCl, pH 8.2, 1 mM Na-EDTA) were used for a two-step alkylation reaction (iodoacetamide (IAM) followed by 4-vinylpyridine (VP)). The first alkylation step involved incubating CprK with 50 mM IAM (final concentration) for 15 min at room temperature. The IAM stock solution was prepared by adding 100 mg of IAM to a solution containing 0.5 M sodium acetate, 2 mM Na-EDTA and incubating at 65 °C until the crystals were dissolved. The mixture was allowed to equilibrate slowly to room temperature. The IAM was protected from light at all times to avoid degradation and production of free iodine radicals, which are potent oxidants. The reaction was quenched by adding 5 ml of cold acetone with 1 n HCl (98:2) followed by centrifuging at 2000 × g for 5 min. The precipitate was washed three times by repeated suspension in a cold acetone with 1 n HCl and H$_2$O solution (98:2:10) and centrifugation as above. After the third wash, precipitated CprK was dissolved in 0.5 ml of Buffer A and reduced by incubating at 37 °C for 30 min with 10 mM DTT. Then the sample was either digested with trypsin overnight at room temperature using a ratio of protein to trypsin of 5:1 and analyzed by mass spectroscopy or treated with a second alkylating agent.

The second alkylation step was performed by adding 2 μl of 9.5 M VP (three times at 20-min intervals) to 0.5 ml of DTT-reduced CprK (above) and incubating for a total of 60 min at room temperature. The protein was precipitated, washed with cold acetone and 1 n HCl, resuspended in Buffer A containing 10 mM DTT as described above, digested with trypsin as just described, and analyzed by mass spectrometry.

Mass Spectrometric Analysis—The trypsin-digested samples were injected onto a C18 PepMap100, 75 μm × 15 cm, 3-μm 100 Å column (LC Packings, Sunnyvale, CA). Tryptic peptides were then separated using a linear gradient elution from 0.3% formic acid in H$_2$O to 0.3% formic acid in acetonitrile at a flow rate of 170 nanoliter/min. The nano-LC (liquid chromatography)/MS/MS analysis of tryptic peptides was performed using a Q-Star XL mass spectrometer (Applied Biosystems Inc.). The masses of tryptic peptides and modifications were matched with the MASCOT search engine. The two tables show the predicted relative molecular masses of the Cys-containing peptides based on their sequences and the experimental relative molecular masses, which were calculated from the experimentally observed $m/z$ values. These tables also show the relative intensities of these peptides, which were calculated from their peak heights at a particular $m/z$ generated from the selected ion chromatogram.

Isothermal Titration Calorimetry Experiments (ITC)—ITC experiments were performed at 25 °C using a VP-ITC isothermal titration calorimeter (MicroCal Inc., Northhampton, MA). The reaction cell (~1.4 ml) was filled with a 0.038 mM solution of purified CprK that had been dialyzed against 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, without DTT, and degassed by vacuum aspiration for 5 min prior to loading. The titrated ligand CHPA (initial concentration, 0.5 mM) was dissolved in the CprK dialysis buffer. The stirring speed was 300 rpm, and the thermal power of 60 injections of 3 μl was recorded every 240 s. Thermogram analysis was performed using Origin 7.0 software supplied with the instrument.

Intrinsic Fluorescence Quenching Experiments—Fluorescence spectroscopy was performed at 20 °C with an OLIS RSM1000F instrument. The starting concentration of CprK (both oxidized and reduced) was 3.0 mM, and fluorescence intensity was measured at 325.6 nm following the addition of CHPA. The excitation was set at 280 nm with an excitation slit of 3.16 nm, an emission slit of 1.24 nm, and a grating of 600 lines/mm. The data were plotted fit to one-site (Equations 1 and 2) and two-site and three-site (Equation 3) models to determine the dissociation constants and to statistically assess the binding model. In these equations, $F$ is the measured fluorescence, and $x$ is calculated from the quadratic Equation 2, where $b$ equals $(E + L + K_{dn})$, i.e. the sum of $E$ (total concentration of enzyme), $L$ (total ligand concentration), and $K_{dn}$ (dissociation constant); and c is the product of the concentrations of $L^*E$. In Equation 3, which describes a bi- or multi-phasic titration curve, $K_{dn}$ represents the dissociation constant(s) (i.e. $K_{d1}$ and $K_{d2}$ for a biphasic curve), $F_0$ is the initial fluorescence, and $F$ and $L$ are defined as for the one-site model (i.e. $F_1$ and $F_2$ designate the amplitudes of the first and second phases of a biphasic titration, respectively).

$$ F = (F_{\text{max}} * K_d)/(x + K_d) \quad (\text{Eq. 1}) $$
$$ x = 0.5[(b + (b^2 + 4c))^{0.5} \quad (\text{Eq. 2}) $$
$$ F = F_0 + \Sigma(F_n * K_{dn}/(K_{dn} + L)) \quad (\text{Eq. 3}) $$
**Redox-active Thiols in CprK**

Nonreducing SDS-PAGE Analysis—Purified proteins from the nickel-nitrilotriacetic acid metal affinity column were dialyzed against a solution of 50 mM Tris, pH 7.5, 300 mM NaCl to remove imidazole. The dialyzed protein was then incubated with 15 mM diamide, 15 mM H₂O₂, and/or 10 mM DTT for 5 min at room temperature. After incubation, nonreducing loading buffer (60 mM Tris-Cl, pH 6.8, 1% SDS, 10% glycerol, 0.01% bromphenol blue) was added, and a 12% SDS-PAGE experiment was run.

**Electrophoretic Mobility Shift Assay (EMSA)**—A 90-bp fragment containing the CprB promoter region was prepared, 5′-end-labeled by [γ-32P]ATP (3000 Ci/mmol; Amersham Biosciences), and used in EMSA experiments as described earlier (9). The reaction mixture contained gel shift binding buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25 mg/ml poly(dI-dC)-poly(dI-dC); final concentrations), CHPA (Sigma-Aldrich), and purified CprK. The reaction was initiated by adding the 32P-labeled DNA fragment, and the mixture was incubated for 20 min at room temperature. The mixtures were separated by electrophoresis, followed by drying and autoradiography as described (9).

**RESULTS**

CprK-Effector Interactions—The results of previous EMSA and ITC experiments showed that CprK binds the effector molecule CHPA with high affinity (3.5 ± 0.4 μM), which promotes DNA binding to a specific palindromic sequence (9). CprK was shown to lose DNA binding activity when it is oxidized by diamide or by extended incubation in air and to regain DNA binding activity when it is incubated with DTT (9).

CprK contains a single tryptophan residue (Trp₁⁰⁶), which is adjacent to Cys₁⁰⁵. When CprK is incubated with CHPA, the fluorescence of this tryptophan residue is quenched, providing a way to measure effector binding affinity. The titration of DTT-reduced CprK with CHPA was fit to Equation 1, which describes a one-site model, and to Equation 3 for two- and three-site models (Fig. 1). The data density was sufficient to rule out one- and two-site models and to support a three-site binding model, based on the goodness of fit parameter (R² = 0.992 for the three-site versus 0.981 for the two-site and 0.919 for the one-site model). The three K_d values are 0.04 μM, 1.5, and 300 μM (Table 1). Approximately 50% of the total fluorescence quenching occurs in the first two phases (about 25% in each of these phases), and the remaining 50% of the intensity is lost in the third phase. At high concentrations of effector, the fluorescence is nearly completely quenched.

Oxidation of CprK could disable either effector or DNA binding. To address the mode of reversible redox inactivation, intrinsic tryptophan fluorescence quenching experiments were performed to compare the affinity of oxidized versus reduced CprK for CHPA. With CprK prepared aerobically and in the absence of a reducing reagent, the total intrinsic fluorescence is ~6.5-fold higher than that of the reduced protein. However, as with the reduced protein, the data best fit a three-site model with dissociation constants of 0.04, 5.9, and 260 μM, which are

**TABLE 1**

|                 | Reduced CprK |                | Oxidized CprK |                |
|-----------------|--------------|----------------|--------------|----------------|
|                 | ITC          | Fluorescence   | ITC          | Fluorescence   |
|                 | K_d (μM)     | Amp (%)        | K_d (μM)     | Amp (%)        |
| 0.02 ± 0.01     | 52 ± 2       | 0.04 ± 0.02    | 22 ± 5       |
| 1.3 ± 0.2       | 48 ± 2       | 1.5 ± 0.7      | 31 ± 4       |
| 300 ± 60        | 47 ± 2       | 4.8 ± 1.6      | 100          |
| 5.9 ± 1.7       | 23 ± 2       |
| 260 ± 17        | 50 ± 2       |

* Amplitude of the phase relative to the total change in fluorescence or heat, expressed as a percentage.
nearly identical to those determined for the reduced protein (Fig. 1 and Table 1).

To further investigate the thermodynamics of effector binding to CprK, ITC experiments were performed with the oxidized and reduced protein. Given the low $K_d$ measured by fluorescence quenching, we collected many data points across the titration curve, especially at low effector concentrations (Fig. 2). Two phases are clearly evident in the ITC profile for the reduced protein, and the two-site model provides a much better fit to the data than a single-site model, based on the chi-square values (1874 for the two-site model versus 3299 for the one-site model). The two $K_d$ values (0.02 ± 0.01 and 1.3 ± 0.2 μM) are similar to the first two $K_d$ values observed by fluorescence quenching experiments, and the two phases have nearly equal amplitudes (Table 1). Our focus was on the tight binding sites, so we did not titrate to high enough effector concentrations to attempt to verify the low affinity (300 μM) binding site detected by fluorescence quenching. We performed ITC studies on the oxidized protein numerous times; however, because this form of CprK is relatively unstable at the high concentration and over the long incubation time required for this experiment, the data were consistently of poorer quality than those for the reduced protein. In this case, we are unable to discriminate between a two-site and a one-site model; therefore, following Occam’s razor, we show the fit to the one-site model in Fig. 2b. Regardless, the ITC data indicate that the oxidized protein contains at least one high affinity site ($K_d = 4.8$ μM), which is in general agreement with the fluorescence quenching data.

In summary, on the basis of fluorescence quenching and ITC studies, we conclude that the oxidized and reduced forms of CprK have two high affinity and one low affinity sites and that CHPA binding is not affected by the redox state of the protein. In both ITC and fluorescence experiments, the phase amplitudes of the two high affinity sites are equivalent, and the amplitude of the low affinity site in the fluorescence experiment equals the sum of the phase amplitudes of the two high affinity sites (Table 1). CHPA binding to the high affinity site(s) is largely entropy driven, with $\Delta S$ values at 310 K of $-9.3 \text{ kJ/mol}$ and $-6.9 \text{ kJ/mol}$ associated with $K_d$ and $K_d$, respectively (Fig. 2).

Because oxidative inactivation of CprK does not measurably alter its interactions with the effector, disulfide bond formation must affect DNA binding affinity. Consistent with earlier results on redox control of CprK activity (9), an EMSA performed with the oxidized protein sample after the ITC experiment showed no detectable DNA binding; however, when 10 mM DTT was incubated with the oxidized CprK-CHPA mixture for 5 min, DNA binding activity was restored (not shown).

The reversibility of the redox inactivation is consistent with the hypothesis that oxidation involves the formation of one or more disulfide bonds, which would disrupt DNA binding by altering the shape of the protein. Oxidation of CprK results in a conformational change that reduces the affinity for DNA, and reduction with DTT restores the native conformation, allowing DNA binding to be restored.

**FIGURE 2.** ITC study of the redox dependence of CHPA binding to CprK in the presence (a) and absence (b) of 10 mM DTT. The x axis indicates the molar ratio of CHPA/CprK. ITC experiments were performed at 25 °C by titrating CHPA into a 1.4-mL reaction cell containing 0.038 mM CprK, 50 mM Tris-HCl, pH 7.5, and 300 mM NaCl. The data (closed squares) were curve fit (line) by thermogram analysis using Origin 7.0 software. Thermodynamic values obtained from the curve fit are, in a, $K_{d1} = 0.019 ± 0.011 \text{ μM}, n1 = 0.90 ± 0.04, \Delta H = 30 \text{ cal/mol}, \Delta S = 1562 ± 20 \text{ cal/mol},$ and $K_{d2} = 1.3 ± 0.2 \text{ μM}, n2 = 0.82 ± 0.03, \Delta S = 23 \text{ cal/mol}, \Delta H = -1160 ± 60 \text{ cal/mol},$ and in b, $K_{d1} = 4.7 ± 1.5 \text{ μM}, n = 1.75 ± 0.07, \Delta S = 20.2 \text{ cal/mol}, \Delta H = -1210 ± 77 \text{ cal/mol},$ where $n$ is the stoichiometry of bound CHPA/CprK monomer.
Redox-active Thiols in CprK

TABLE 2
Mass spectrometry results of air-oxidized and reduced CprK alkylated with IAM

| Peptide          | Sequence                          | Air-oxidized | Reduced |
|------------------|-----------------------------------|--------------|---------|
|                  |                                   | IAM          |         |
|                  |                                   | No. a | M_r (calc) | M_r (expt) | Intensity | No. a | M_r (calc) | M_r (expt) | Intensity |
| 1–25             | AMSAEGLKDPCGIIPDSFPEEK            | ND  | ND         | ND        | 0        | 1    | 2757.30    | 2757.33    | 65     |
| 105–114          | TCFWSRGLCR                        | 0    | 1272.53    | 1270.46   | 4000     | 2    | 1386.57    | 1386.49    | 4928    |
| 157–167          | LFYECQSGQK                        | 1    | 1331.61    | 1330.57   | 1886     | 1    | 1330.62    | 1330.57    | 234     |
| 198–203          | VLACLK                            | 0    | 645.39     | 645.37    | 0        | ND   | ND         | ND        | ND      |
| 198–204          | VLACLK                            | 0    | 773.48     | 773.46    | 337      | ND   | ND         | ND        | ND      |
| 1–25 + 198–203   | AMSAEGLKDPCGIIPDSFPEEK + VLACLK  | 0    | 3343.67    | 3343.56   | 175      | ND   | ND         | ND        | ND      |
| 1–25 + 198–204   | AMSAEGLKDPCGIIPDSFPEEK + VLACLK  | 0    | 3471.76    | 3471.64   | 227      | ND   | ND         | ND        | ND      |

a Number of cysteine residues modified.
b Relative molecular mass based on the matched peptide sequence.
c Experimental m/z factored by z.
d ND indicates that this peptide was not detected under these conditions.

TABLE 3
Mass spectrometry results of air-oxidized and reduced CprK modified by two-step alkylation

| Peptide          | Sequence                          | Air-oxidized | Reduced |
|------------------|-----------------------------------|--------------|---------|
|                  |                                   | IAM          |         |
|                  |                                   | No. a | M_r (calc) | M_r (expt) | Intensity | No. a | M_r (calc) | M_r (expt) | Intensity |
| 1–25             | AMSAEGLKDPCGIIPDSFPEEK            | 1    | 2757.30    | 2757.33   | 150      |
| 10–25            | DFCGIIIPDSFPEEK                   | 1    | 2757.30    | 2757.33   | 150      |
| 198–203          | VLACLK                            | 1    | 702.41     | 702.42    | 945      |
| 198–204          | VLACLK                            | 0    | 750.45     | 750.45    | 200      |

a Number of cysteine residues modified.
b Relative molecular mass based on the matched peptide sequence.
c Experimental m/z factored by z.
d ND indicates that this peptide was not detected under these conditions.

disulfide bonds. Another possibility is that one of the cysteine residues is oxidized to a sulfinate or sulfenate, which also would also be reversed by treatment with DTT (13). To distinguish between these various possibilities, chemical alkylation and mass spectrometric experiments were performed.

Alkylation of Cysteine Residues and Mass Spectrometric Analysis of CprK—CprK from D. dehalogenans contains five cysteine residues: Cys11, Cys105, Cys111, Cys161, and Cys200. To assess whether any of these residues are involved in disulfide bond formation, CprK samples were prepared by exposure to air, oxidation with diamide, or reduction with 10 mM DTT. The CprK samples were denatured by urea to expose all of the Cys residues, subjected to a two-step alkylation procedure, and then analyzed by mass spectrometry. The first alkylation step involved treatment of the urea-denatured protein with IAM, which labeled Cys residues that were present in the thiolate form to generate S-carboxamidomethylcysteine. The second alkylation step involved reduction with DTT, followed by treatment with VP. Cys residues that were initially present in a disulfide bond would not be labeled by IAM during the first alkylation step; however, after reduction with DTT, those residues would react with VP and undergo conversion to S-(4-vinylpyridinyl)ethylcysteine. CprK samples were digested with trypsin and analyzed by mass spectrometry after either the first or second alkylation step.

The results of the one-step and two-step alkylation procedures are shown in Tables 2 and 3. In these tables, the number of IAM modifications is listed beside the peptide identification. The predicted molecular mass of each peptide, based on its sequence, was compared with the experimentally determined mass. The tables also include the intensity value, which does not measure the absolute quantity of a peptide but quantifies the relative abundance of a particular peptide under similar liquid chromatography/MS conditions.

When CprK samples were reduced with 10 mM DTT and alkylated by IAM, mass spectrometric analysis revealed that Cys11, Cys105, Cys111, and Cys161 were modified with carboxamido labels. Thus, in the presence of DTT, these four Cys residues were initially present in the thiolate form (Table 2). Although when samples were analyzed after this one-step IAM alkylation experiment, a peptide covering Cys200 was not detected, all five cysteine residues were modified by IAM in the first step of the two-step alkylation of reduced CprK (Table 3). In summary, these data indicate that DTT-reduced active protein contains all five cysteine residues in the thiol or thiolate form.

When the air-oxidized protein was analyzed by the one-step alkylation reaction, a peptide containing S-carboxamidomethyl-labeled Cys161 (peptide 157–167) was recovered in high amounts (Table 2), indicating that this residue was in the thiol state.
form under both reducing (above) and oxidizing conditions. None of the other four Cys residues (Cys¹¹, Cys¹⁰⁵, Cys¹¹¹, and Cys²⁰⁰) of air-oxidized CprK were labeled by IAM. The MS analysis did not indicate the presence of sulfenate, sulfinate, or sulfonate modification of any Cys-containing peptides. An unlabeled peptide 105–114 containing Cys¹⁰⁵ and Cys¹¹¹ was identified whose mass was lower by 2.06 Da than predicted, suggesting the loss of two hydrogen atoms (Fig. 3A), which would be expected for a peptide containing a disulfide bond. A smaller amount of this peptide contained IAM modification, whereas in the DTT-reduced samples, the doubly IAM-modified peptide 105–114 was present in much higher proportion than the unmodified one (supplemental Fig. S1). These results indicate that, in the air-oxidized protein, Cys₁⁶¹ is a reduced thiol(ate), and Cys¹⁰⁵ and Cys¹¹¹ are linked by a disulfide bridge. The situation with Cys¹¹ and Cys²⁰⁰ is addressed below.

In agreement with the results of the one-step alkylation protocol, when air-oxidized CprK was subjected to the two-step procedure, peptide 105–114 contained two S-(4-pyridylethyl) modifications (Table 3). A small proportion of this peptide was found with both modifications (S-carboxymethyl and S-(4-pyridylethyl)); however, the peptide containing the double modification by VP was 6-fold more abundant. Therefore, both the single and double alkylation results indicate that Cys¹⁰⁵ and Cys¹¹¹ form a disulfide bond in air-oxidized CprK. This disulfide bridge is most likely intramolecular because these residues are close in the primary sequence. Furthermore, we could not locate a peak with the mass expected for two covalently linked 105–114 peptides during analysis of the single alkylation experiment. The two-step procedure also results in IAM modification of the peptide containing Cys¹⁶¹, with a smaller amount of VP modification. Based on the results with the one-step procedure, we assume that the minor amount of Cys¹⁶¹ labeled by VP simply reflects incomplete IAM labeling, although we cannot rule out a minor proportion of Cys¹⁶¹ being involved in a disulfide link with another residue.

MS analysis of the peptides containing Cys¹¹ and Cys²⁰⁰ was more complicated. When the oxidized CprK sample was treated with only IAM, we did not observe a peptide containing Cys¹¹ (Table 2). However, as shown in the last two rows of Table 2, we observed two peaks with masses corresponding to the sum of two tryptic peptides, one containing unmodified Cys¹¹ (peptide 1–25) and the other containing unmodified Cys²⁰⁰ (peptides 198–203 and 198–204) (Fig. 3B). The difference between these peaks is that peptide 198–203 has one Lys residue at the C terminus, whereas peptide 198–204 ends in Lys-Lys, because of incomplete trypsin digestion. These results indicate that peptides 1–25 and 198–203 are cross-linked, as are peptides 1–25 and 198–204. The combined masses of both cross-linked peptides were 2 Da lower than that predicted for the sum of the individual peptides, which like peptide 105–111 indicates the loss of two hydrogen atoms. Unlike the 105–114 peptide, this 2-Da mass difference is not evident in Table 2, because these cross-linked peptides were identified by assuming the loss of two hydrogen atoms and manually searching for peptides with these predicted masses. These combined results indicate that the 198–203/204 and 10–25 peptides are cross-linked by disulfide bonds.

When air-oxidized CprK was analyzed by the two-step alkylation experiment, both the 198–203 and 10–25 peptides were modified by VP (Table 3), providing additional evidence for a disulfide bridge between Cys¹¹ and Cys²⁰⁰. However, these experiments do not permit distinction between a disulfide cross-link between two subunits (intermolecular) or within one
**Redox-active Thiols in CprK**

The results of SDS-PAGE experiments with the C200A variant were similar to those with C11S. In the absence (supplemental Fig. S2, lane 10) or presence (lane 11) of DTT, the protein remained predominantly in the monomeric state. When treated with diamide (lane 12), monomeric CprK was predominant, but there was a small proportion of dimeric and higher oligomeric forms (albeit, a larger proportion than with C11S). These results support the mass spectrometric results implicating Cys\(^{11}\) and Cys\(^{200}\) in intermolecular disulfide bond formation.

**DISCUSSION**

CprK is the transcriptional regulator of the eight-gene cpr regulon of desulfitobacteria, which encodes the pathway for coupling the reductive dehalogenation of chlorinated aromatics to ATP synthesis. Earlier in vivo and in vitro DNA binding studies demonstrated that CprK only functions under reducing conditions, that oxidation of CprK prevents effector-triggered transcriptional activation of the cprB gene, and that reduction of the oxidized protein reverses oxidative inactivation (9). Two questions addressed here are: which residues are responsible for redox regulation of CprK transcriptional activity and whether oxidative inactivation occurs at the level of effector or DNA binding.

Fig. 5 proposes a two-tier mode of regulation of dehalorespiration. One level of regulation involves a redox equilibrium shown horizontally, whereas the other involves effector binding, depicted vertically. The work described here shows that oxidative inactivation involves two classes of disulfide bonds that are formed when CprK is exposed to air, diamide, or \(\text{H}_2\text{O}_2\). Based on mass spectrometric studies, all five cysteine residues (Cys\(^{11}\), Cys\(^{105}\), Cys\(^{111}\), Cys\(^{161}\), and Cys\(^{200}\)) are in the reduced thiol(ate) state. On the other hand, studies of the oxidized protein identified one peptide containing Cys\(^{105}\) and Cys\(^{111}\) and two cross-linked peptides containing Cys\(^{11}\) and Cys\(^{200}\) that were two mass units lighter than would be predicted based on their sequences. These peptides did not undergo alkylation until CprK had been reduced with DTT. Complementing the mass spectrometric data are SDS-PAGE studies, which show that the oxidized native protein is a dimer, whereas the C11S and C200S variants remain predominantly in their monomeric forms. Therefore, as shown in Fig. 5, we conclude that Cys\(^{11}\) and Cys\(^{200}\) form an intermolecular disulfide bond, and Cys\(^{105}\) and Cys\(^{111}\) form an intramolecular disulfide linkage. In this model, the redox switch involves interconversion of all four Cys residues between their transcriptionally active reduced state and an inactive oxidized state. Further studies are required to determine the specific roles of the intermolecular versus the intramolecular cross-links in controlling DNA binding.

The oxidized C200A variant retains a slight ability to dimerize, suggesting the possibility that, when the sulfur of Cys\(^{200}\) is absent, Cys\(^{11}\) can form a disulfide link with another residue (perhaps Cys\(^{161}\)). Further studies are underway with various double mutants to test this possibility.

Among the CprK homologs, Cys\(^{105}\) and Cys\(^{200}\) are highly conserved. Furthermore, Cys\(^{105}\) is equivalent to Cys\(^{122}\) in the redox regulator, *Escherichia coli* FNR (Fig. 6), where it ligates one of the iron atoms in a [4Fe-4S] cluster that is required for

**FIGURE 4. Nonreducing SDS-PAGE.** Lanes 2–5 contain wild-type CprK, lanes 6–9 contain the CprK C11S variant, and lanes 10–13 contain the C200S variant. Lane 1, protein standards; lanes 2, 6, and 10, no DTT; lanes 3, 7, and 11, 50 mM DTT; lanes 4, 8, and 12, 5 mM diamide; lanes 5, 9, and 13, 5 mM diamide plus 50 mM DTT.

Subunit (intramolecular). Nonreducing SDS-PAGE analysis was used to discriminate between these structural options.

**SDS-PAGE Analysis of Disulfide-linked Subunits**—In parallel with the mass spectrometric results, nonreducing SDS gel electrophoresis experiments were performed on samples prepared under different redox conditions (Fig. 4). To prevent reductive cleavage of disulfide bonds during sample preparation, DTT was omitted from the SDS sample denaturant solution. This experiment should allow the identification of redox conditions that stabilize the monomeric (27947.1 Da) as well as any intermolecular cross-linked dimeric species (55,894 Da). Because the samples are treated with SDS, noncovalently associated subunits will dissociate into monomers.

When wild-type CprK was incubated with 10 mM DTT, the sample was found predominantly in a monomeric state, yet a weak band appeared at the position expected for a dimer at ~50 kDa (supplemental Fig. S2). When the protein was reduced with 50 mM DTT, only the monomeric protein was observed (lane 3). Thus, the DTT-reduced protein does not contain a detectable amount of intermolecularly cross-linked dimeric protein.

When CprK was stored in the absence of DTT (lane 2) or oxidized with diamide (lane 4) or \(\text{H}_2\text{O}_2\) (supplemental Fig. S2), it was found to be predominantly as a dimer. Higher oligomeric states were also evident. Treatment with DTT converted the oxidized dimer back to the monomeric state (lane 5). Complementary results using dynamic light scattering show that the oxidized protein exhibits a much higher degree of polydispersity than the reduced protein, which behaves like a relatively monodisperse dimeric unit (not shown).

In the presence of 10 mM DTT, the C11S variant of CprK, like the wild-type protein, was predominantly in the monomeric state with a small amount of dimer (supplemental Fig. S2) and was converted quantitatively to the monomeric state when incubated with 50 mM DTT (lane 7). Unlike the wild-type protein, when the C11S variant was stored in the absence of DTT (lane 6) or when it was oxidized with diamide (lane 8) or \(\text{H}_2\text{O}_2\) (supplemental Fig. S2), it remained predominantly in the monomeric state, with very small amounts of dimeric and higher oligomeric states. Therefore, in the C11S variant, intermolecular disulfide bond formation is clearly disfavored.

The oxidized C200A variant ... double mutants to test this possibility.

Among the CprK homologs, Cys\(^{105}\) and Cys\(^{200}\) are highly conserved. Furthermore, Cys\(^{105}\) is equivalent to Cys\(^{122}\) in the redox regulator, *Escherichia coli* FNR (Fig. 6), where it ligates one of the iron atoms in a [4Fe-4S] cluster that is required for
O2 sensing (14–16). Cys\(^{105}\) is also conserved in an Fnr-like protein from Lactobacillus casei called FlpA, which contains only two Cys residues that, like CprK, appear to undergo reversible oxidation from the dithiol to the disulfide state in the presence of O2 (17).

As shown in Fig. 5, besides redox regulation, effector binding to CprK controls transcriptional activity. It would be inefficient for cells to synthesize the cpr gene cluster when the haloaromatic substrate is absent. For proteins like CprK in the CRP/FNR family, binding of the ligand to the effector domain triggers interactions of the helix-turn-helix DNA-binding domain with a specific upstream regulatory DNA sequence. The conformational changes responsible for ligand-induced DNA binding have been reviewed and appear to involve a hinge region between the two domains and the repositioning of a long helix at the dimer interface (18, 19).

We tested whether oxidative inactivation occurs at the level of effector or of DNA binding. The results of ITC and fluorescence quenching studies clearly show that the oxidized and reduced states of CprK have identical affinity for CHPA. Both the oxidized and reduced forms of CprK have two high affinity and one low affinity sites. It is interesting that in both ITC and fluorescence experiments, the phase amplitudes of the two high affinity sites are equivalent, and the amplitude of the low affinity site in the fluorescence experiment equals the sum of the phase amplitudes of the two high affinity sites. Given that the protein is a dimer, this suggests that there are two types of sites: one low affinity site (\(K_d = \sim 0.3 \text{ mM}\)) that is present in both subunits and is not influenced by interactions with the other subunit, and a high affinity site that is nonequivalent (\(K_d\) values of \(\sim 0.05\) and \(\sim 5 \mu\text{M}\)) in the two subunits. CHPA binding to the high affinity site(s) is largely entropy driven, with \(\Delta S\) values at 310 K of \(-9.3\) and \(-6.9 \text{ kJ/mol}\) associated with \(K_d^1\) and \(K_d^2\), respectively. The corresponding \(\Delta G\) values are \(-10.9\) and \(-8.1 \text{ kJ/mol}\). Furthermore, these thermodynamic data suggest that there is a larger decrease in degrees of freedom (\(\Delta \Delta S = 2.4 \text{ kJ/mol}\)) on binding the second mol of CHPA than the first, suggesting the possibility of negative cooperativity. However, there is insufficient data to rule out the possibility that the two subunits are inherently nonequivalent. Thus, Fig. 5 simply depicts two independent conformational changes that control transcriptional activity: one is a redox switch, and the other is an effector-triggered remodeling of CprK to enable DNA binding. In Fig. 5, we have not shown the low affinity site but speculate that this site might be in the DNA-binding domain, because a low affinity CAMP-binding site is found near the DNA-binding site of CRP (20). The model shown in Fig. 5 depicts our working model that either Cys\(^{11}\) or Cys\(^{200}\) may be in the DNA-binding domain because formation of the Cys\(^{11}\)–Cys\(^{200}\) intermolecular disulfide bond disables DNA binding without affecting substrate binding. If Cys\(^{11}\) is in the DNA-binding domain and Cys\(^{200}\) is in the effector domain (or vice versa), oxidation of CprK would lead to a major conformational change that would prevent DNA binding, independent of the effector concentration.

One important question is why redox regulation of transcription would occur in D. dehalogenans, which is an anaerobic or microaerophilic organism. Efforts to address this question directly are underway. Several arguments supporting the physiological relevance of the redox regulation can be considered. A variety of anaerobic microbes have been found to have pathways for dealing with oxygen and oxidative stress. For example, functional cytochrome oxidases are found in some “strict anaerobes” that allow them to respire on oxygen (21), perhaps as a defense mechanism. Furthermore, because desulfitobacteria are microaerophiles, they occupy niches that are often exposed to oxygen. Thus, redox regulation of CprK would be one of many mechanisms to recognize and deal with oxygen and oxidative stress. The genome sequence of Desulfitobacterium hafniense (genome.jgi-psf.org/draft_microbes/desha/desha.home.html) includes genes encoding a variety of oxygen stress response enzymes, including superoxide dismutases, catalase, and alkylhydroperoxidases.

In conclusion, our studies reveal a strategy for limiting expression of a redox-sensitive metabolic pathway by using a
Redox-active Thiols in CprK

thiol-based redox switch in the transcription factor. A number of transcriptional activators and DNA-binding proteins contain redox sites that allow them to activate or repress the transcription of genes depending on oxygen concentrations and/or redox state. Among these are thiol-based regulatory switches (13). In the case of the dehalorespiration pathway, the thiol/disulfide switch prevents transcription of the cpr gene cluster when cells are subjected to oxidative conditions, because activity of the reductive dehalogenase (CprA) requires two iron-sulfur clusters and a corrinoid cofactor (11, 22–25), both of which are subject to oxidative inactivation (26–29).

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Addendum—While this paper was under review, a manuscript was submitted and accepted describing the crystal structure of the D. hafniense CprK in its oxidized state and the D. dehalogenans CprK in its reduced state (30). The structure reveals the intermolecular Cys$^{111}$–Cys$^{200}$ disulfide bond (the D. hafniense protein lacks Cys$^{111}$, so the Cys$^{105}$–Cys$^{111}$ disulfide bond is not present in this protein), which unfavorably positions the helix-turn-helix domain for binding DNA.

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