Characterization of the B$_{12}$- and Iron-Sulfur-containing Reductive Dehalogenase from Desulfitobacterium chlororespirans*§

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The United Nations and the U.S. Environmental Protection Agency have identified a variety of chlorinated aromatics that constitute a significant health and environmental risk as “priority organic pollutants,” the so-called “dirty dozen.” Microbes have evolved the ability to utilize chlorinated aromatics as terminal electron acceptors in an energy-generating process called dehalorespiration. In this process, a reductive dehalogenase (CprA), couples the oxidation of an electron donor to the reductive elimination of chloride. We have characterized the B$_{12}$ and iron-sulfur cluster-containing 3-chloro-4-hydroxybenzoate reductive dehalogenase from Desulfitobacterium chlororespirans. By defining the substrate and inhibitor specificity for the dehalogenase, the enzyme was found to require an hydroxyl group ortho to the halide. Inhibition studies indicate that the hydroxyl group is required for substrate binding. The carboxyl group can be replaced by other functionalities, e.g. acetyl or halide groups, ortho or meta to the chloride to be eliminated. The purified D. chlororespirans enzyme could dechlorinate an hydroxylated PCB (3,3',5,5'-tetra-chloro-4,4'-biphenyldioly) at a rate about 1% of that with 3-chloro-4-hydroxybenzoate. Solvent deuterium isotope effect studies indicate that transfer of a single proton is partially rate-limiting in the dehalogenation reaction.

There are at least three distinct enzyme classes that can remove the halogen substituent from organic compounds: the hydrolases, the methyltransferases, and the reductive dehalogenases. In this report we focus on an enzyme in the reductive dehalogenase class. The hydrolytic dehalogenases, which replace the chloro group with an hydroxyl functionality, are by far the best studied. Hallmarks of this enzyme class are the use of an aspartate residue as a nucleophile to form a covalent enzyme-substrate adduct and a general base residue to facilitate the hydrolysis step. High resolution structures are available for the 4-chlorobenzoyl-CoA (3), haloacid (4), and haloalkane (5) dehalogenases. The methyltransferase class uses vitamin B$_{12}$ as a cofactor in a group transfer reaction (6). The reductive dehalogenases replace the chloro functionality with a hydride equivalent. Two types of reductive dehalogenases include dehalorespiration-linked metalloenzymes that couple the reduction of the chlorinated hydrocarbons to ATP synthesis via a chemiosmotic mechanism (2, 7) and members of the glutathione S-transferase class, which feature a glutathione-dependent substitution reaction in their mechanism (8).

This report focuses on a dehalorespiration-linked reductive dehalogenase. Members of the dehalorespiratory class of reductive dehalogenases have several interesting features. Enzymes have been described that can dehalogenate a discrete set of aliphatic and aromatic compounds. Perhaps the most interesting and potentially important reactions are the microbial reductive dechlorination of polychlorinated biphenyls (PCBs) and their oxygenated metabolites, the hydroxylated PCBs (OH-PCBs), by anaerobes (9, 10) like Desulfitobacterium dehalogenans (11). Microbes have been isolated from contaminated soils and sediments that can catalyze removal of meta, para, and ortho chlorines of PCB mixtures by reductive dehalogenation (12). Furthermore, some microbes have evolved the capacity to gain energy from the reductive dehalogenation reaction (13, 14). For example, Dehalospirillum multivorans generates a membrane potential and/or a pH gradient associated with the transfer of electrons from the electron donor (H$_2$ or pyruvate) to the chlorinated organic (15). In all but one case, the enzymes, encoded by the cprA gene, that catalyze the reductive dehalogenation are vitamin B$_{12}$- (or cobamide-) and iron-sulfur-containing enzymes. In at least some desulfitobacteria, the cprA gene is found within a cluster of genes that includes a membrane anchor (CprB), a membrane-associated regulatory protein (CprC), a DNA binding protein (CprK), and several chaperones (CprT, CprD, and CprB) (16). In this report we will typically refer to the dehalogenase as CprA.

Two general mechanisms have been proposed for the dehalorespiring reductive dehalogenases (modified from Ref. 2) (see

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1 The abbreviations used are: PCB, polychlorinated biphenyl; OH-PCB, hydroxylated PCB; GC-MS, gas chromatography-mass spectrometry; PCE, tetrachloroethene; SKIE, solvent kinetic isotope effect.
Fig. 1. Mechanistic models for the corrinoid iron-sulfur reductive dehalogenase. This was modified from a previous study (2). The two pathways, A (dashed arrows) and B (solid arrows), differ in the roles of cobalamin. In A, an organocobalt adduct is formed; in B, Co(I) donates an electron to the aromatic ring. Possible transition states for a proton transfer reaction are shown in the box at the upper right. Although the mechanism and the transition state diagram assume a radical intermediate, there is sparse evidence for it and there are other viable possibilities. R, designates an additional functional group on the ring.

Because dehalorespiring bacteria were discovered only recently, many questions about the dehalogenation mechanism remain unanswered. Are organocobalt adducts analogous to those in B12-dependent methyltransferases; whereas, in the other (path B), the corrinoid serves as an electron donor. In path A, the halide is eliminated as the aryl-Co(III) intermediate is formed. Path B resembles the Birch reduction of hydroxylated aromatics in which a radical anion is formed. Because the corrinoid is the most electron-withdrawing substituent on the ring, the carbon to which it is bonded would bear the highest charge density in the radical anion. This position, therefore, would be most activated toward protonation, assuming that the proton transfer occurs before or concerted with chloride elimination, as shown in the upper transition state diagram in Fig. 1. Yet one could imagine proton addition from solvent occurring after chloride departure, as in the lower transition state structure.

Experimental Procedures

Organism and Growth Conditions—D. chlororespirans (ATCC 700175) was grown with agitation on reduced anaerobic medium (ATCC 2035), containing 20 mM pyruvate, 2 mM 3-chloro-4-hydroxybenzoate, and 1 g of yeast extract per liter (17). The 13-liter cultures were incubated in a 14-liter fermentor at 37 °C for 4 days using an 80% N2/20% CO2 headspace gas. Addition of 20 mM pyruvate and 2 mM 3-chloro-4-hydroxybenzoate on the second day of incubation increased the optical density at 600 nm (A600) to 0.7–0.8. D. chlororespirans cells were harvested with a continuous flow CEPA centrifuge at 16,000 × g, yielding 1.4 g/liter wet weight of cells, which were stored at −80 °C.

Purification of CprA—14.6 g of cells was suspended in 35 ml of buffer A (100 mM potassium Pi, pH 7.2, 2 mM dithiothreitol) plus 40 units/ml DNase I and sonicated (30 min, 30 s pulse on, 30 s pulse off) under anaerobic conditions (95% N2/5% H2). The cell-free extract was separated into a membrane fraction and a soluble fraction by centrifugation for 4 h at 105,000 × g and 4 °C. The membrane fraction was resuspended in 20 ml of buffer A supplemented with 3% (w/v) Triton X-100 and 20% glycerol and incubated at 4 °C for 2 h under anaerobic conditions. The insoluble fraction was removed from this preparation by centrifugation for 2 h at 105,000 × g and 4 °C. The supernatant contained CprA, which was further processed.

All chromatographic steps were performed in an anaerobic chamber with a N2/H2 (95%/5%) gas phase. The membrane fraction was diluted with an equal volume of buffer B (50 mM potassium Pi, 0.1% Triton X-100, 20% glycerol, and 2 mM dithiothreitol) at pH 6.2 and loaded on a 5 × 7 cm Q-Sepharose column (Bio-Rad) equilibrated with the same buffer. The column was eluted with a 600-ml linear gradient from 0 to 500 mM NaCl in buffer B at a flow of 3 ml/min.

Fractions containing the highest dechlorination activity were pooled and diluted with an equal volume of buffer B (same as buffer A, but at pH 8.0) and applied to a High Q-Sepharose column (2.5 × 7 cm) (Bio-Rad) equilibrated with buffer B. The enzyme was eluted with a 120-ml linear gradient from 0 to 500 mM NaCl in buffer B at a flow rate of 1.3 ml/min.

Fractions containing dehalogenase activity were pooled and mixed with an equal volume of buffer B, pH 8.0, and applied to a High Q-Sepharose column (Econo-Pac cartridges, 5 ml) (Bio-Rad) equilibrated with the same buffer. The enzyme was eluted with a 60-ml linear gradient from 0 to 600 mM NaCl in buffer B at a flow rate of 1 ml/min.

The protein concentration was determined according to Bradford (18) with bovine serum albumin as a standard. The pH and temperature optima were determined in buffer B at pH values ranging from 5.1 to 8.7 and temperatures from 25 to 70 °C.

Dehalogenase activity in every fraction was assayed by TLC, as described below. Dehalogenase activity in the combined fractions after each step of purification was estimated spectrophotometrically, as described below.

TLC—The assay mixture, which contained 0.3 ml of buffer B (pH 7.6), 40 µl of 15 mM methyl viologen, 50 µl of 0.2 M titanium citrate, 10 µl of 50 mM 3-chloro-4-hydroxybenzoate, and 10–100 µl of sample, was anaerobically incubated for 30 min at 57 °C and then quenched by adding 50 µl of 0.2 M perchloric acid and centrifuged at 10,000 × g for 1 min. The clear supernatant was extracted with 0.5 ml of diethyl ether, and the organic phase was spotted on Silica Gel AL SIL G/UV plates (Whatman Ltd.). The mobile phase consisted of toluene:acetic acid:water (6:7:3, v/v). The separated products were visualized by use of a UV lamp and identified by their relative mobility (Rf) values, which are...
0.46 and 0.32 for 3-chloro-4-hydroxybenzoate and 4-hydroxybenzoate, respectively.

Spectrophotometric Dehalogenase Assay and Determination of Kinetic Parameters—Dehalogenation of 3-chloro-4-hydroxybenzoate and other chlorinated substrates was measured spectrophotometrically at 578 nm and 37 °C by following the oxidation of reduced methyl viologen (ε578 = 9.7 mM⁻¹ cm⁻¹) (19). The 0.5-mI assay mixture contained 0.5 mM chlorinated compound and 0.3 mM methyl viologen in buffer B at pH 7.6. The methyl viologen was reduced by adding titanium(III) citrate until the absorbance at 578 nm reached 2.6. The linearity of the absorbance value to 2.6 OD units at 578 was confirmed. The reaction was initiated by adding 5–50 μl of dehalogenase (the final enzyme concentration was 20–150 nm). One unit of activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of chlorinated substrate, which equals the oxidation of 2 μmol of reduced methyl viologen, per minute. Steady-state kinetic parameters were determined from plots of the initial rates of dehalogenation versus substrate concentration (5 μM to 10 mM), which were fit to the Michaelis-Menten equation using SigmaPlot. The inhibition constant (Ki) was determined by fitting experimental data to the following equation: 

\[ v = V_{max}S/(S + K_i) \]

where V\(_{max}\) and K\(_i\) correspond to the parameters for 3-chloro-4-hydroxybenzoate dechlorination without inhibitor, and \( i \) stands for inhibitor concentration.

Measuring Dehalogenation of a Hydroxy-PCB—In studies of the dehalogenation of hydroxy-PCBs, the 0.5-mI assay mixtures contained 20 mM titanium(III) citrate, 0.6 mM 3,3',5,5'-tetrachloro-4,4'-biphenyldiol, and 22–28 μg/ml dehalogenase in 50 mM Tris-HCl buffer, pH 7.6. After incubating at 57 °C for various times, the reaction was quenched by exposing to air and extracting with dichloromethane. The organic phase was analyzed for the parent compound (3,3',5,5'-tetrachloro-4,4'-biphenyldiol) and dechlorination products by gas chromatography-mass spectrometry (GC-MS). Injections (1 μl) were made in the splitless mode on a Varian 3400CX GC coupled to a Saturn 3 ion trap mass spectrometer using an 8200 Autosampler and helium as the carrier gas. A fused silica GC column (30 m × 0.25 mm i.d.) coated with 0.25-μm DB-XLB (Agilent Technologies) was used to separate compounds of interest. The GC was programmed as follows: 70 °C (2-min hold); ramp to 280 °C at 6 °C/min (3-min hold); total run time of 40 min. The injector was initially held at 70 °C and then ramped ballistically to 280 °C at 200 °C/min. The transfer line and manifold temperature were held isothermal at 280 °C and 240 °C, respectively. The ion trap was operated in the electron impact full scan mode (m/z 50–350) at 1.0 cycles/s and was turned on 8 min after sample injection. Repeated injections of a 100 μg/ml solution of the parent 3,3',5,5'-tetrachloro-4,4'-biphenyldiol (m/z 288, 290) and a dichloro-biphenyldiol (m/z 232, 234) were made to determine the electron impact mass spectrum and to assess the response and the change in GC-MS sensitivity, if any, through the analytical run. No variability in relative response and/or mass spectra of parent/daughter compounds was observed. Two additional GC peaks that appeared to be related to the parent compound were detectable in all extracts, including a trichloro-biphenyldiol (m/z 189, 288, 290) and a dichloro-biphenyldiol (m/z 232, 234). The relative abundances and molar concentrations of these dichloro- to tetrachloro- compounds were estimated assuming a uniform MS response equal to that determined for the parent compound and the appropriate molecular masses. The purity of the parent 3,3',5,5'-tetrachloro-4,4'-biphenyldiol was reported by the supplier to be 95%. We estimated the purity at 92%, with as much as 7% trichloro-biphenyldiol as impurity. Less than 1% of the dichloro-biphenyldiol and no dichloro-biphenyldiol was found in the original solution. The mean relative amounts of the parent/dechlorination products were estimated by gel filtration to be 198 kDa, which is more than three times higher than the apparent mass estimated by SDS-page electrophoresis (65 kDa) (24). Similar results were obtained for the PCE reductive dehalogenase from Dehalobacter restrictus (25). As shown by gel filtration and ultracentrifugation studies of heterodisulfide reductase, which is also isolated in the presence of Triton, much of the molecular mass is contributed by tightly associated detergent (26).

Properties of CprA—The N-terminal amino acid sequence of CprA is AATDFTNYVPGKQGKQSKLL. This sequence is similar to those of dehalogenases from other Desulfotobacteria, e.g. D. hafniense (55%), D. dehalogenans (45%), D. viet-1 (45%), and D. PCE-I (45%) (Fig. 3). Because the first amino acid is Ala, the protein probably has been post-translationally processed as have the other dehalogenases, which contain a 40-amino acid signal sequence that is removed as the protein matures and binds to the bacterial membrane (27). All the published sequences of the reductive dehalogenases that correspond to an active protein from Desulfotobacteri amines contain a conserved NYVPG sequence (Fig. 3).

After treating the 3-chloro-4-hydroxybenzoate reductive dehalogenase with potassium cyanide at 90 °C and removing the protein precipitate, the typical visible light absorption spectrum of dicyano-cob(III)alamin (21) is observed (inset in Fig. 4). Using an extinction coefficient at 580 nm of 10.13 mM⁻¹ cm⁻¹ (21), the corrinoid content is 0.84 ± 0.05 mol cobrinol/mol of subunit. Besides cobalamin, a monomeric unit of the enzyme buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol, pH 9.1. The transferred protein band was stained with 0.1% Amido black, excised, and sequenced by automated N-terminal Edman degradation using an ABI-494 Procise sequencer.

RESULTS AND DISCUSSION

Purification of CprA—The 3-chloro-4-hydroxybenzoate reductive dehalogenase (CprA) was purified from D. chlororespirans cells that were grown on pyruvate as a carbon and electron source and 3-chloro-4-hydroxybenzoate as an electron acceptor. More than 90% of the enzyme activity was found in the membrane fraction and was extracted with the nonionic detergent Triton X-100 (3% v/v) (Table I). The tetrachloroethylene dehalogenase from Desulfitobacterium strain PCE-S (7) and the 3-chloro-4-hydroxyphenylacetate reductive dehalogenase from Desulfitobacterium hafniense (23) are also membrane-associated and must be extracted and purified in the presence of detergent.

The purification scheme for 3-chloro-4-hydroxybenzoate dehalogenase is summarized in Table I. During purification, the specific activity increased 181-fold to a final value of 15.4 units/mg of protein. SDS-gel electrophoresis analysis of purified dehalogenase revealed a single band of ~50-kDa molecular mass (Fig. 2). The requirement for detergent to stabilize the protein prevents an accurate determination of the native molecular mass. For example, the size of the monomeric PCE reductive dehalogenase from D. strain PCE-S was determined by gel filtration to be 198 kDa, which is more than three times higher than the apparent mass estimated by SDS-page electrophoresis (65 kDa) (24). Similar results were obtained for the PCE reductive dehalogenase from Dehalobacter restrictus (25).

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### Table I

| Step                  | Protein | Activity | Specific activity | Purification factor |
|-----------------------|---------|----------|------------------|---------------------|
| Cell-free extract     | 987     | 76.1     | 0.085            | 1                   |
| Soluble fraction      | 584     | 4.6      | 0.008            | 3                   |
| Membrane fraction     | 295     | 69.3     | 0.281            | 4                   |
| Q-Sepharose, pH 6.2   | 16.7    | 56.3     | 3.4              | 40                  |
| Q-Sepharose, pH 8.0   | 3.6     | 34.5     | 9.6              | 113                 |
| High Q, pH 8.0        | 1.6     | 24.6     | 15.4             | 181                 |

D. chlororespirans Dehalogenase

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contains 7.6 g-atm iron and 1.0 g-atm Co, based on metal analysis, and 8.1 mol of acid-labile sulfide. These results suggest that the dehalogenase from *D. chlororespirans* contains one corrinoid and two iron-sulfur clusters per monomeric unit. Except for the enzyme from *Desulfomonile tiedjei*, which is apparently a heme enzyme (28), the reductive dehalogenases so far purified from dehalorespiring organisms contain cobamide and iron-sulfur clusters.

The *D. chlororespirans* enzyme is completely inhibited when the enzyme is incubated with 450 μM propyl iodide. Approximately 50% of its initial activity is regained when the enzyme is exposed to light. This characteristic of many cobamide-containing enzymes is implicated in dehalogenation of chlorinated phenols by this bacterium. Given the potential use of reductive dehalogenases in environmental bioremediation, it is important to establish their substrate specificity. CprA exhibits Michaelis-Menten kinetics with a variety of chlorinated phenols (Fig. 5 and Table II). The substrate range of the purified dehalogenase (Table II) agrees well with that reported for the *D. chlororespirans* cells (17) and its membrane fraction (34), indicating that a single enzyme is involved in dehalogenation of chlorinated phenols by this bacterium. We compared the specificity parameters (kcat/Km) for different chlorinated aromatics that can undergo dehalogenation, including 3-chloro-4-hydroxybenzoate, 3,5-chloro-4-hydroxybenzoiate, 3-chloro-4-hydroxyphenylacetate, 2,3-dichlorophenol, 2,6-dichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol (Table II). Lack of an hydroxyl group in the position ortho to the chlorine substituent prevents dehalogenation. For example, 3- or 4-chlorobenzoate and 2,6-, 2,4-, or 3,5-dichlorobenzoate are not metabolized. Chlorine does not replace the requirement for the hydroxyl group, because 3,4-dichlorobenzoate is not dehalogenated.

Barring one exception, the enzyme only metabolizes aro-
mation compounds; tetrachloroethene, trichloroethane, and 2,4-dichloropropene are not dechlorinated. Trichloroacetate undergoes dehalogenation with a specific activity of 18.8 μmol/mg/min, which is similar to that observed with 3-chloro-4-hydroxybenzoate. Yet, unlike any of the aromatic substrates, ~10% of the methyl viologen is consumed during the reaction with trichloroacetate. One possible explanation for this finding is that the viologen radical may react with and possibly form an adduct with a radical intermediate either on the enzyme, as in cobalamin-dependent methionine synthase (37), or the substrate. Because this behavior is restricted to trichloroactic acid, it suggests that a substrate rather than an enzyme-based intermediate reacts with the blue viologen cation radical. Further studies are required to determine the reason for the consistent loss of the methyl viologen when the enzyme utilizes trichloroacetate. Alternatively, CprA-catalyzed dehalogenation of trichloroacetate follows a different mechanism than that used for reduction of chlorinated phenols.

Why is an ortho hydroxyl group required for all the chlorinated aromatic substrates? It could be required for binding or could participate in the chemistry of the dehalogenation reaction. An hydroxyl group ortho to the halogen atom is required for the tetrachloroethylenoxide reductive dehalogenase from Sphingomonas chlorophenolica, which is a member of the glu-tathione S-transferase superfamily (38, 39). In this case, the ortho-hydroxyl group participates in elimination of an enzyme-glutathione adduct that forms subsequent to the dechlorination step.2

The requirement for an hydroxyl group ortho to the chlorine that undergoes elimination represents a limitation for the bioremediation of PCBs and related xenobiotics lacking an ortho hydroxyl group. If the ortho-hydroxyl group is involved in binding, such an hydroxyl group will also be required for inhibitors of CprA activity. Consistent with the requirement for binding, 4-hydroxybenzoate, the product of the 3-chloro-4-hydroxybenzolate dehalogenation reaction, and 3-hydroxybenzoate are competitive inhibitors (Ki = 1.7 and 12.8 mM, respectively), whereas 3-chlorobenzoate is not an inhibitor (Table III). 4-Hydroxybenzoate also inhibits the dehalogenation of 3-chloro-4-hydroxyphenylacetate and 2,4,6-trichlorophenol, with similar Ki values (1.6 mM) as that of 3-chloro-4-hydroxybenzoate, which is consistent with competitive inhibition. Similarly, 3,6-dichlorosalicylate (3,6-dichloro-2-hydroxybenzoate) inhibits the enzyme but 3,6-dichlorobenzoate and 3,6-dichloro-2-methoxybenzoate (dicamba) do not.

Is the need for an hydroxyl group in the ortho position to the chlorine a general attribute of corrinoid iron-sulfur-containing aromatic dehalogenases? This requirement has also been reported for the enzymes that have been purified from D. dehalogenans (35) and D. hafniense (23). Yet, the heme-containing dehalogenase from D. tiedjei catalyzes meta dechlorination (28). Because a major role of the hydroxyl group appears to be in substrate binding, it is likely that corrinoid-dependent enzymes containing modified substrate binding pockets will be found that can catalyze dehalogenation with the hydroxyl group at other positions of the ring and even lacking the hydroxyl group. D. hafniense (23, 40) and Desulfotobacterium frappieri (41) cells can catalyze ortho, meta, and para dehalogenation; however, the corrinoid-containing dehalogenase from D. hafniense that that has been purified apparently requires an ortho-hydroxyl group (23) and none of the dehalogenases from D. frappieri have been purified. Purification and characterization of dehalogenases with different substrate specificities will help in understanding the roles of corrinoid and of the hydroxyl group in the dehalogenation of chlorinated phenols. Furthermore, because the major role for the hydroxyl group is in binding, we surmise that it may be possible to engineer a dehalogenase like the D. chlororespirans enzyme that lacks this requirement.

With the D. chlororespirans dehalogenase, an hydroxyl group ortho to the chlorine is necessary, but not sufficient for binding to the enzyme, because 2-chlorophenol is not a substrate or inhibitor (Ki > 1 mM). When there is a carboxyl or acetyl group meta to the chlorine (3-chloro-4-hydroxybenzoate or 3-chloro-4-hydroxyphenylacetate), the compound is a sub-

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**Table II**

| Substrate                          | Vmax | kcat | KM  | kcat/KM | Relative kcat/KM |
|------------------------------------|------|------|-----|---------|-----------------|
| 3-Chloro-4-hydroxybenzoate         | 14.8 | 12.3 | 12.4 | 0.99    | 100.0           |
| 3,5-Dichloro-4-hydroxybenzoate     | 37.9 | 31.6 | 12.3 | 2.57    | 259.6           |
| 3-Chloro-4-hydroxyphenylacetate     | 5.5  | 4.6  | 310  | 0.015   | 1.5             |
| 2,3-Dichlorophenol                 | 10.2 | 8    | 334  | 0.025   | 2.6             |
| 2,6-Dichlorophenol                 | 5.1  | 4.5  | 1460 | 0.005   | 0.3             |
| 2,4,6-Trichlorophenol              | 12.6 | 10.5 | 160  | 0.065   | 6.5             |
| Pentachlorophenol                  | 1.94 | 1.6  | 390  | 0.0044  | 0.4             |
| Tetrachlorodiol<sup>1</sup>        | 0.12 | 0.1  | ND  | ND      | ND              |

<sup>1</sup> No dechlorination was detected with 3-chlorobenzoate, 4-chlorobenzoate, 2,4-dichlorobenzoate, 3,4-dichlorobenzoate, 3,5-dichlorobenzoate, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 3,5-dichlorophenol, 3,5-dichloro-2-hydroxybenzoate, 3,5-dichloro-2-methoxybenzoate (dicamba), 2-chloro-3-hydroxyphenol (2-chloro-resorcinol), or 2-chloro-4-hydroxyphenol (chlorohydroquinone).

<sup>2</sup> Dechlorination rate corresponds to 0.6 m/M 3,3′,5,5′-tetrachloro-4,4′-trihydroxyphenylidol.

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**Table III**

| Inhibitor                          | Ki (μM) |
|------------------------------------|---------|
| 3-Chlorobenzoate                   | <1 μM   |
| 3,5-Dichlorobenzoate               | <1 μM   |
| 2-Chlorophenol                     | ND<sup>2</sup> |
| Tetrachloroethene                  | ND<sup>2</sup> |
| 2,4-Dichloropropene                | <1 μM   |
| 3-Chloro-4-methylbenzoate          | ND<sup>2</sup> |
| 2-Chloro-3-hydroxyphenol, 2-chlororesorcinol | >1 μM |
| 2-Chloro-4-hydroxyphenol, chlorohydroquinone | >1 μM |
| 3,6-Dichloro-2-hydroxybenzoate     | 140 ± 30 μM |
| 3-Hydroxybenzoate                  | 12.8 ± 0.9 mM |
| 4-Hydroxybenzoate                  | 1.7 ± 0.3 mM |

<sup>1</sup> Inhibition constant (Ki) was determined by a nonlinear fit of experimental data to a competitive inhibition equation using SigmaPlot (see "Experimental Procedures").

<sup>2</sup> ND, no alteration in the Ki or Vmax of 3-chloro-4-hydroxybenzoate.

<sup>3</sup> 1M indicates that the compounds were weakly inhibitory, but the K values were near 1 mM and had high errors.

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2 S. D. Copley, personal communication.
substrate. Interestingly, placing another chlorine in the ortho or meta position relative to the chlorine, is sufficient for reactivity, albeit only 6.5 or 0.3% relative to the para-carboxyl. The ortho-chloro derivative (2,3-dichlorophenol) is approximately as reactive as the meta-acetyl (3-chloro-4-hydroxyphenylacetate). However, 2,4-dichlorophenol is neither a substrate nor an inhibitor. Adding additional halides to the ring only marginally enhances reactivity, because the specificity factors for 2,4,6-trichlorophenol and pentachlorophenol are only 20- and 1.3-fold higher, respectively, than 2,6-dichlorophenol. Even though 3,6-dichlorosaliclylate (3,6-dichloro-2-hydroxybenzoate) has an ortho hydroxyl and a carboxyl group, it is not a substrate, but an inhibitor with a \( K_m \) value (140 \( \mu \)M) 10-fold greater than the \( K_m \) for 3,5-dichloro-4-hydroxybenzoate (12 \( \mu \)M). The chloro group is less important for binding than the para-carboxyl or the ortho-hydroxyl, because para- and meta-hydroxybenzoate are inhibitors.

\textit{Dehalogenation of Hydroxy-PCB—}The conditions for dehalogenation of OH-PCBs and PCBs by anaerobic bacteria have been reviewed previously (12). The ability of the \textit{D. chlororespirans} dehalogenase to remove chlorine from aromatic rings containing an ortho-hydroxyl group and another substituent in the position meta to the halide led us to test whether hydroxylated polychlorinated biphenyls (OH-PCBs), which contain a meta-phenyl ring, can serve as substrates. The OH-PCBs, formed by mono- and dioxygenases, are the main metabolites of PCBs. When CprA was incubated with 3,3',5,5'-tetrachloro-4,4'-biphenylyl (tetrachlorobiphenylyl) under standard assay conditions, trichloro- and dichlorobiphenylyl were detected as the products (m/l 189, 288, 290 and \( m/l \)) 139, 238, 240, respectively) (Fig. 6a). There is increased product formation with higher amounts of enzyme, demonstrating enzyme-dependent degradation of the hydroxy-PCB. The rate of this reaction in the presence of 0.6 mM substrate is 0.12 \( \mu \)mol/mg/min at 57 °C (Fig. 6b), which is \(-0.3\%\) of the maximal rate with saturating levels of 3-chloro-4-hydroxybenzoate at the same temperature. This represents the first example of OH-PCB dehalogenation by a pure enzyme and suggests the possibility of using purified dehalogenases to aid in PCB biodegradation. The requirement for the hydroxyl group for binding to the enzyme assures that PCBs themselves will not serve as inhibitors. However, OH-PCBs in most contaminated sites are a diverse mixture of many congeners, some of which might inhibit and others which might react with the enzyme. No dehalogenation was observed with 2',3',4',5',6'-tetrachloro-3-biphenyl, 2',4',6'-trichloro-4-biphylol, and 5-chloro-2-biphenylyl, which is consistent with the requirement for the chloro group to be ortho to the hydroxyl group.

\( ^2 \)H\(_2\)O Solvent Kinetic Isotope Effects—The \( k_{cat} \) value for dehalogenation of 3-chloro-4-hydroxybenzoate by the \textit{D. chlororespirans} CprA is 2.3-fold higher in 100% \( H_2O \) than in 100% \( \text{D}_2O \) (Fig. 7). A fully rate-limiting proton transfer would be expected to give a \( ^2 \)H\(_2\)O solvent kinetic isotope effect (SKIE) of \(-4\); therefore, an intramolecular proton transfer reaction is partially rate-limiting in the dehalogenase reaction. The linearity of the proton inventory plot indicates that a single proton is in flight in the transition state. These SKIE studies indicate that cleavage of an \( H-O \) bond occurs in the rate-determining step of the dehalogenase reaction. The haloalkane dehalogenase from \textit{Xanthobacter autotrophicus} GJ10 exhibits a \( ^2 \)H\(_2\)O SKIE of \(-1.9\) on \( k_{cat} \); however, this enzyme catalyzes replacement of the halide with hydroxide instead of "hydrdide." The SKIE for haloalkane dehalogenase appears to originate from a slow conformational change (42) in the enzyme associated with solvation of the halide ion product as it leaves the active site (43). We suggest that the SKIE for the reductive dehalogenase from \textit{D. chlororespirans} might originate from the rate-limiting deprotonation of a water molecule, because deuterium from solvent is incorporated specifically into the carbon of 2,5-dichlorobenzoate that undergoes dehalogenation by cell extracts of \textit{D. tiedjei} (44). A similar situation occurs in carbonic anhydrase, where \( ^2 \)H\(_2\)O SKIEs between 2 and 3.8 are observed (36, 45–48). The SKIE studies seem most consistent with path B of Fig. 1, in which the partially rate-limiting proton transfer step occurs after an electron is transferred to the aromatic ring and before or concerted with the departure of the chloride ion. It is not expected that protonation of the neutral radical following homolysis would be a rate-limiting step, as in path A. The SKIE could also derive from solvation of the halide ion to facilitate elimination from the active site, as has been proposed for the haloalkane dehalogenase (43).

\textit{Conclusion—}We have characterized the corrinoid- and iron-sulfur-dependent reductive dehalogenase (CprA) from \textit{D. chlororespirans}. Steady-state kinetic studies of substrate and inhibitor specificities indicate that the enzyme only utilizes substrates with an hydroxyl group ortho to the halide plus...
another functional group meta to the chlorine (carboxyl > acetyl > chloro). Because these requirements appear to be mainly important for substrate binding, we anticipate that enzymes can be designed or isolated that have the ability to dehalogenate environmentally significant chloroaromatics. The studies represent the first description of a purified dehalogenase that can catalyze the dechlorination of an hydroxy- 

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Characterization of the $\text{B}_12^-$ and Iron-Sulfur-containing Reductive Dehalogenase from *Desulfotobacterium chlororespirans*

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