Purification and Molecular Characterization of *ortho*-Chlorophenol Reductive Dehalogenase, a Key Enzyme of Halorespiration in *Desulfitobacterium dehalogenans*.

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*orthocha*rophenol reductive dehalogenase of the halorespiring Gram-positive *Desulfitobacterium dehalogenans* was purified 90-fold to apparent homogeneity. The purified dehalogenase catalyzed the reductive removal of a halogen atom from the *ortho* position of 3-chloro-4-hydroxyphenylacetate, 2-chlorophenol, 2,3-dichlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol, pentachlorophenol, and 2-bromo-4-chlorophenol with reduced methyl viologen as electron donor. The dechlorination of 3-chloro-4-hydroxyphenylacetate was catalyzed by the enzyme at a V\textsubscript{max} of 28 units/mg protein and a K\textsubscript{m} of 20 \textmu M. The pH and temperature optimum were 8.2 and 52 °C, respectively. EPR analysis indicated one [4Fe-4S] cluster (midpoint redox potential (E\textsubscript{m}) = −440 mV), one [3Fe-4S] cluster (E\textsubscript{m} = +70 mV), and one cobalamin per 48-kDa monomer. The Co(I)/Co(II) transition had an E\textsubscript{m} of −370 mV. Via a reversed genetic approach based on the N-terminal sequence, the corresponding gene was isolated from a *D. dehalogenans* genomic library, cloned, and sequenced. This revealed the presence of two closely linked genes: (i) cprA, encoding the *ortho*-chlorophenol reductive dehalogenase, which contains a twin-arginine type signal sequence that is processed in the purified enzyme; (ii) cprB, coding for an integral membrane protein that could act as a membrane anchor of the dehalogenase. This first biochemical and molecular characterization of a chlorophenol reductive dehalogenase has revealed structural resemblance with haloalkene reductive dehalogenases.

Anaerobic bacteria that are able to conserve metabolic energy from the dechlorination of chlorinated compounds have gained a lot of attention because of their role in bioremediation of contaminated sites and the novel respiration pathways they possess (1). Halorespiring bacteria have been found within the groups of low G + C Gram-positives, green nonsulfur bacteria, and \delta- and \epsilon-proteobacteria. These bacteria can use chloroalkenes, e.g. tetrachloroethene (PCE)\textsuperscript{1} and trichloroethene (TCE) or chloroaromatic compounds such as chlorophenols or 3-chlorobenzoate as the terminal electron acceptor.

The halorespiratory pathway of anaerobic PCE degradation has been studied in some detail. A key enzyme in this respiratory pathway is the PCE reductive dehalogenase, which catalyzes the reductive removal of a chlorine atom from PCE and TCE. A 58-kDa PCE reductive dehalogenase was purified from *Dehalosporillum multivorans*, which contains cobalamin and probably two iron-sulfur clusters (2). Cloning and sequencing of the corresponding cprA gene revealed the presence of an additional open reading frame, *pceB*, being cotranscribed with *cprA* and coding for an 8-kDa membrane-spanning protein (3). The PCE reductive dehalogenases isolated from *Dehalobacter restrictus* (60 kDa) and *Desulfobacterium frappieri* strain PCE-S (65 kDa) resemble the enzyme from *Dehalosporillum multivorans* with respect to cofactor content and catalytic properties (4, 5). EPR analysis of the *D. restrictus* enzyme confirmed the presence of cobalamin and two [4Fe-4S] clusters. All chloroalkene reductive dehalogenases characterized up to now are monomeric and either membrane-bound or membrane-associated.

Enzymes involved in chloroaryl respiration have been studied in *Desulphonile tiedjei* and *Desulfobacterium* species (6–8). However, no further molecular characterization of these enzymes was reported.

We investigated *ortho*-chlorophenol dechlorination in *Desulfitobacterium dehalogenans*. This organism is able to couple the reductive dechlorination of different *ortho*-chlorinated phenolic compounds to growth with lactate, pyruvate, formate, or hydrogen as electron donor (9, 10). Comparison of biomass yields on pyruvate and different electron acceptors indicated that chlorophenol dechlorination in *D. dehalogenans* is an energy-yielding process (11). This study for the first time describes the purification and characterization of the catalytic subunit of the *ortho*-chlorophenol reductive dehalogenase (*o*-CP dehalogenase) from *Desulfitobacterium dehalogenans*. Its redox properties were studied by EPR spectroscopy, and the corresponding *cprA* gene was cloned and characterized, revealing structural resemblance with haloalkene reductive dehalogenases.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions—*D. dehalogenans*** strain JW/UI-DC1 (DSM 9161) was cultivated under anaerobic conditions (100% N\textsubscript{2} gas phase) in 25-liter vessels containing 20 liters of basal medium as described by Neumann et al. (12), supplemented with 0.2% yeast extract, 20 mM lactate sodium salt, 20 mM 3-chloro-4-hydroxyphenylacetate, 50 mM NaHCO\textsubscript{3}, and trace elements and vitamin

or DCP, dichlorophenol; PCR, polymerase chain reaction; ORF, open reading frame; *o*-CP dehalogenase, *ortho*-chlorophenol reductive dehalogenase.

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\textsuperscript{1} The abbreviations used are: PCE, tetrachloroethene; TCE, trichloroethene; Cl-OHPA, 3-chloro-4-hydroxyphenyl acetate; CP, chlorophenol; DCP, dichlorophenol; PCR, polymerase chain reaction; ORF, open reading frame; *o*-CP dehalogenase, *ortho*-chlorophenol reductive dehalogenase.

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF115542.

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solution as recommended by the German Collection of Microorganisms. The 20-liter cultures were incubated at 37 °C for 2 days. After 1 day of incubation, 250 ml of 2 mM NaOH was added to the culture to avoid acidification of the medium.

*Escherichia coli* XL-1-Blue (Stratagene) was used as a host for cloning vectors. The strain was grown in Luria Bertani medium at 37 °C, and ampicillin was added at 100 µg/ml when appropriate. The cloning vectors pUC18 and pUC19 were purchased from Amersham Pharmacia Biotech, and pHON38201 was obtained from Monsanto.

**Preparation of Cell Extracts**—Lacto-exponential phase cultures of *D. dehalogenans* were harvested by continuous flow centrifugation at 16,000×g (Biofuge 28RS, Heraeus Separations), which yielded 1.6 g of concentrated cells/liter of culture. The concentrated cells were stored at −20 °C. 8 g of cells was resuspended in 8 ml of buffer 1, consisting of 100 mM potassium phosphate (KP), pH 7.5, and 2.5 mM dithiothreitol. A few crystals of DNase I were added to the cell suspension. Cells were broken by sonication (Vibra, Sonic Materials Inc.) under anaerobic conditions. The cell debris was removed by centrifugation for 5 min at 20,000 × g. The supernatant was incubated for 10 min in the presence of 0.5 M KCl and 0.2% Triton X-100 and then separated into a membrane fraction and a soluble fraction by centrifugation for 90 min at 140,000 × g and 4 °C. The membrane fraction was resuspended in 8 ml of buffer 1 supplemented with 1% Triton X-100 and 20% glycerol and incubated for 60 min under anaerobic conditions at 4 °C. The insoluble fraction was removed from this preparation by centrifugation for 60 min at 140,000 × g and 4 °C. The solubilized enzyme fraction was stored under a N2 gas phase at 4 °C.

**Column Chromatography**—All chromatographic steps were performed by fast protein liquid chromatography (Amersham Pharmacia Biotech) in an anaerobic chamber with N2/H2 (95%/5%) gas phase. The Triton X-100 concentration of the sample was raised to 3% before it was applied to a column to prevent protein aggregation. The solubilized enzyme preparation was loaded on a Q-Sepharose column (2.2 × 8.9 cm) (Amersham Pharmacia Biotech) equilibrated with buffer A (50 mM KP, pH 6.0, 0.1% (w/v) Triton X-100, 20% glycerol, and 1 mM dithiothreitol). The column was eluted with a 75-ml linear gradient from 0 to 300 mM NaCl in buffer A at a flow of 2.5 ml/min. The NaCl concentration of the medium was increased to 100 mM by linear gradient elution from 0 to 400 mM NaCl in buffer A at a flow of 1.0 ml/min at a NaCl concentration of 180 mM.

Combined fractions containing dechlorinating activity were mixed with an equal volume of buffer B (50 mM Tris-HCl, pH 7.8, 0.1% w/v Triton X-100, 20% glycerol, and 1 mM dithiothreitol) and applied on a Mono Q column equilibrated with the same buffer. The enzyme activity was eluted with a 40-ml linear gradient from 0 to 400 mM NaCl in buffer B and a flow rate of 1.0 ml/min at a NaCl concentration of 180 mM.

**Enzyme Assay**—Chlorophenol reductive dehalogenase activity was assayed spectrophotometrically at 578 nm and pH 7.8 by monitoring the reduction of ferricyanide as described by Massey and Hemmerich (19). The enzyme was completely reduced in 45 min by deazaflavin/EDTA-mediated light reduction as described by Massey and Hemmerich (19). Deazaflavin was synthesized according to Janda and Hemmerich (20). One unit is defined as the amount of enzyme that catalyzed the reduction of 1 µmol of reduced methyl viologen per minute. The same specific activity of the enzyme was determined by continuous flow centrifugation at 4 °C.

**DNA Isolation, Manipulation, and Oligonucleotides**—Chromosomal DNA from *D. dehalogenans* was isolated as follows. Protoplasts were prepared from 12 ml of culture (A600 = 0.4) as described by van Asseldonk et al. (22), recovered at 13,000 × g for 2 min, and resuspended in 100 µl of THMS buffer (30 mM Tris-HCl, pH 8.0, and 3 mM MgCl2 in 25% sucrose). After the addition of 400 µl of 50 mM Tris-HCl (pH 8.0) containing 5 mM EDTA, 50 mM NaCl, and 0.5% SDS, chromosomal DNA was purified through successive steps of phenol/chloroform extraction and recovered by ethanol precipitation.

Plasmid DNA was isolated from *E. coli* by using the alkaline lysis method, and standard DNA manipulations were performed according to established procedures (23) and the manufacturers’ instructions. Enzymes were purchased from Life Technologies, Roche Molecular Biochemicals, or New England Biolabs. Oligonucleotides and [α-32P]dATP were obtained from Life Technologies and Amersham Pharmacia Biotech, respectively. Prehybridization and hybridization were performed at 65 and 50 °C, respectively. Posthybridization washes were conducted at 40 °C.

Oligonucleotides used in this study were BG 444 (5'-GCG AGA/AGC ATG AATG AA/ACT TA/CTG GTI CCI GGI CCI AAI CT/CT GCI GCI GCI ATT/CT CII AA/AGG AGG ATT/AT TTI GGI CCI GT-3'), nucleotides 644–703), BG 485 (5'-GGG AGC TGG TGG AGA ATT G-3'), nucleotides 427–436, and BG 475 (5'-GGG AGC TGG TGG AGA ATT G-3'), nucleotides 1366–1384). In order to restrict the extent of degeneration for BG 444, insone (1) was used at 3- or 4-fold degenerated positions.

**DNA Amplification by Inverse PCR**—Inverse PCR (24) was performed as follows. Chromosomal DNA was digested with HincII and ligated at a concentration of 0.5 ng/µl of self-ligated DNA was used as the template in a 25-µl PCR reaction containing the following; 2 µl of each primer; 2.25 mM MgCl2, 200 µM dATP, dCTP, dGTP, and dTTP; and 1 unit of Expand® Long Template enzyme mixture (Roche Molecular Biochemicals). The DNA was amplified using the GeneAmp® PCR System 2400 (Perkin-Elmer). After preheating to 94 °C for 2 min, 35 cycles were performed, consisting of denaturation at 94 °C for 20 s, primer annealing at 50 °C for 30 s, and elongation at 68 °C for 3 min. After 10 cycles, the elongation time was extended with 20 s/cycle. A final extension of 7 min at 68 °C was included. PCR products were purified from agarose gel by Gene Clean (Bio 101) and cloned into pMON38201 cut with XcmI.

**DNA Sequencing and Sequence Analysis**—DNA sequencing was performed using a Li-Cor DNA sequencer 4000L. Plasmid DNA used for sequencing reactions was purified with the QIAprep Spin Miniprep kit (Qiagen GmbH). Reactions were performed using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech). Infrared labeled oligonucleotides were purchased from MWG Biotech. Sequences and similarity searches and alignments were performed using the BLAST 2.0 program (25) (NCBI) and the programs Clustal X and GeneDoc (26), respectively.

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2 K. B. Nicholas and H. B. J. Nicholas, unpublished communication.
**RESULTS**

**Purification and Characterization of o-CP Dehalogenase**—o-Chlorophenol reductive dehalogenase was purified under strict anaerobic conditions from the membrane fraction of *D. dehalogenans* grown on lactate and 3-chloro-4-hydroxyphenylacetate (Table I). The specific activity increased 90-fold upon purification and amounted to 28 units/mg protein with reduced methyl viologen as an artificial electron donor. The purified enzyme had a pH and temperature optimum of 8.1 and 52 °C, respectively. At 30 °C, the enzyme showed Michaelis-Menten kinetics for Cl-OHPA. The *K_m* for this chlorinated substrate was determined to be 20 μM at a methyl viologen concentration of 0.3 mM. Cl-OHPA showed no inhibitory effect up to 10 mM, which was the highest concentration used. Several halogenated compounds were tested as possible alternative substrates for o-CP dehalogenase. Activity of o-CP dehalogenase was observed with 2-CP, 2,3-dichlorophenol (2,3-DCP), 2,4-DCP, 2,6-DCP, and pentachlorophenol as substrate (Table II). 3-CP, 4-CP, 2,5-DCP, 2-fluoro-4-chlorophenol, PCE, and TCE were dechlorinated at a rate below the detection limit (0.12 units/mg).

**Organization of the cpra Locus**—The N-terminal amino acid sequence of the cpra gene product of *D. dehalogenans* revealed a 2.7-kilobase region of the DNA region cloned and sequenced. A third open reading frame, ORF1, starts at nucleotide 1958. The N-terminal amino acid sequence of the putative enzyme preparation revealed one band of approximately 48 kDa (Fig. 1). An accurate determination of the native size of the enzyme was not possible due to the high concentration of detergent needed to prevent protein aggregation (data not shown).

**SDS-polyacrylamide gel electrophoresis analysis** of the purified enzyme preparation revealed one band of approximately 48 kDa (Fig. 1). An accurate determination of the native size of the enzyme was not possible due to the high concentration of detergent needed to prevent protein aggregation (data not shown).

**N-terminal Sequence, Cloning, and Sequencing of the cpra Locus**—The N-terminal amino acid sequence of the cpra gene product of *D. dehalogenans* was determined and revealed the sequence NH_2-AETMNYVPGPTNARSKLRPVH-IDE. A 59-bp 256-fold degenerated oligonucleotide (BG 444) was designed based on the sequence of the first 20 N-terminal amino acids. Southern blot analysis of EcoRI–HindIII-digested chromosomal DNA of *D. dehalogenans* revealed a 2.7-kilobase fragment that hybridized strongly to radiolabeled BG 444. This fragment was cloned in E. coli using EcoRI–HindIII-digested pUC18, resulting in pLUW910. Sequence analysis of the HindIII–HincII 1.8-kilobase fragment of pLUW910 revealed the determined N-terminal amino acids immediately downstream of the HindIII site, indicating that pLUW 910 lacks the translation start of the gene of interest. Therefore, the divergent primer pair BG 458/BG 475 was used to specifically amplify the pLUW910 upstream flanking fragment in an inverse PCR from HincII-digested chromosomal DNA. To ensure determination of the correct nucleotide sequence, three independently obtained PCR products were cloned yielding pLUW912a-c. From these, HincII deletion clones were prepared, giving the corresponding pLUW913a-c. Fig. 2 shows a restriction map of the DNA region cloned and sequenced.

### Table I

| Sample              | Protein | Activity | Yield | Specific activity | Purification factor |
|---------------------|---------|----------|-------|------------------|---------------------|
| Cell-free extract   | 419.0   | 129      | 100   | 308              | 1.0                 |
| Membrane fraction   | 240.0   | 101      | 78    | 423              | 1.4                 |
| Solubilized fraction| 91.0    | 109      | 84    | 1210             | 4.0                 |
| Q-Sepharose fraction| 21.1   | 101      | 78    | 4780             | 15.0                |
| Mono Q, pH 6.0, fraction| 4.4 | 64       | 50    | 14,612           | 47.0                |
| Mono Q, pH 7.8, fraction| 2.1 | 59       | 46    | 27,872           | 90.0                |

*One unit of activity is defined as the oxidation of 2 μmol of reduced methyl viologen.

### Table II

**Substrate specificity profile of purified o-CP dehalogenase**

| Substrate                | Specific activity | Ratio (compared with Cl-OHPA) |
|--------------------------|-------------------|-------------------------------|
| CI-OHPA                  | 12.0              | 100                           |
| 2-Bromo-4-chlorophenol    | 24.3              | 202                           |
| 2,3-DCP                  | 15.5              | 129                           |
| 2,4-DCP                  | 4.2               | 35                            |
| 2,6-DCP                  | 0.8               | 7                             |
| PCP                      | 0.2               | 2                             |
| 2-CP                     | 0.2               | 2                             |

- - 97.4 (phosphatase b)
- - 66.2 (serum albumin)
- - 45.0 (ovalbumin)
- - 31.0 (carbonic anhydrase)
- - 21.5 (trypsin inhibitor)

**Fig. 1.** 12% SDS-polyacrylamide gel electrophoresis with the purified ortho-chlorophenol reductive dehalogenase of *D. dehalogenans* (5 μg) in lane 1. Molecular size markers are shown in lane 2. The arrow indicates the purified protein band. The gel was stained with Coomassie Brilliant Blue R-250.
N-terminal residues of CprA comprise a leader sequence that is cleaved off upon maturation of the protein, leaving a mature 405-amino acid polypeptide with a calculated molecular mass of 45,305 Da. The leader sequence contains an RR motif characteristic for a large number of mainly periplasmic proteins binding different redox cofactors (28). These twin arginine signal sequences (consensus(S/T)RRXFLK) are thought to play a major role in the maturation and translocation of such proteins. As all twin arginine signal sequences, the CprA leader sequence shows the structural characteristics of standard Sec signal sequences. Furthermore, the established cleavage site -VANA | AETM- follows the “−1−3 rule” of von Heijne (29).

The D. dehalogenans CprA sequence reveals the presence of an extended cluster of cysteine residues (Cys330−Cys387, Fig. 3). The first group of four cysteines Cys330−Cys340 is identical to the consensus sequence of bacterial ferredoxin type clusters (CXXCXXCXXCP; Ref. 30), including the conserved proline at position 341. The second cluster shows the same conserved residues (Cys380−Pro388) but lacks the first cysteine. The B12 binding motif DXHXXG-(41)-SxL-(26−28)-GG, as it was determined for a subset of B12-dependent enzymes (31), is not present in CprA.

Upstream of cprA, a second potential gene, cprB, was found, that could encode a 103-amino acid polypeptide with a calculated molecular mass of 11,517 Da. The predicted cprB gene product does not exhibit significant similarities with any known proteins present in the data bases. A hydrophilicity plot indicates the presence of three membrane-spanning helices (Fig. 4). Following the positive-inside rule for integral membrane proteins (32), the N terminus of this polypeptide is predicted to point outward, whereas the C-terminal part is located at the cytoplasmic face of the membrane. CprB and cprA are separated by only 12 nucleotides. Neither transcription termination nor initiation signals are present between the two genes. Preliminary experiments suggest co-transcription of both genes (data not shown).

Cobalamin Involved in Electron Transfer in o-CP Ddehalogenase—Cobalt in biological systems occurs in oxidation states 3+, 2+, and 1+. Only the Co2+ 3d7-system is half-integer spin
and, therefore, readily detectable in EPR spectroscopy. In cobalamin, the Co$^{2+}$ is low spin $S = 1/2$. The EPR of D. dehalogenans o-CP dehalogenase, as isolated, exhibits a signal characteristic for Cob(II)alamin in the base-on form and a weak, near isotropic, $S = 1/2$ signal around $g = 2$ indicative of [3Fe-4S] (see below).

Previously, it was found that full chemical reduction of another reductive dehalogenase, the PCE reductase from D. restrictus, could not be achieved with dithionite (4). Therefore, we used the light-induced strongly reducing system of deazaflavin plus EDTA. Prolonged illumination resulted in a clear EPR spectrum that is dominated by a signal with $g$ values of 2.05, plus EDTA. Prolonged illumination resulted in a clear EPR spectrum that is dominated by a signal with $g$ values of 2.05.

![FIG. 5. EPR spectra of D. dehalogenans o-CP dehalogenase. Trace A, the [4Fe-4S] signal from enzyme fully reduced by illumination with visible light for 50 min in the presence of 20 μM deazaflavin and 2 mM EDTA. Base-on Cob(II)alamin can be detected as a minor component in trace A. Trace B, the base-off Cob(II)alamin signal from enzyme reoxidized by 0.5-min anaerobic incubation with 2 mM Cl-OHPA. Trace C, the [3Fe-4S] signal from enzyme fully oxidized by anaerobic incubation with 2 mM potassium ferricyanide for 5 min. EPR conditions were as follows: microwave frequency, 9.41 GHz; microwave power 5 milliwatts (as follows: microwave frequency, 9.41 GHz; microwave power 5 milliwatts); modulation frequency, 100 kHz; modulation amplitude, 0.63 millitesla; temperature, 9.5 K (trace A), 30 K (trace B), 15 K (trace C).](Image 77x476 to 269x729)

![FIG. 6. EPR-monitored redox titration of the metal centers in D. dehalogenans o-CP dehalogenase. +, [3Fe-4S]$^{4+}$; •, Cob(I)alamin; ▄, [4Fe-4S]$^{4+}$. Starting from a redox potential of −130 mV, the sample was reduced by substoichiometric additions of dithionite and oxidized by substoichiometric additions of ferricyanide, both in the presence of a mixture of redox mediators covering the full potential axis. Amplitudes are given as a percentage of maximal signal intensities. The latter correspond to enzyme fully oxidized by excess ferricyanide or enzyme fully reduced by light/deazaflavin/EDTA. These extreme forms have undefined potentials and are presented as points on the vertical borders. EPR conditions were as in Fig. 5. The solid traces are fits to the Nernst equation assuming single electron transfer.](Image 324x575 to 538x729)

The purified o-CP dehalogenase contains one [4Fe-4S] clus-

**DISCUSSION**

Ortho-Chlorophenol reductive dehalogenase is the terminal reductase involved in the halorespiratory chain of D. dehalogenans. Here we describe the purification and molecular characterization of this key enzyme and its gene cprA. This membrane-associated enzyme mediates the electron transfer from a yet unidentified electron donor to the halogenated substrate. The substrate spectrum of the purified enzyme was similar to that reported for resting cells, indicating that a single enzyme is involved in dehalogenation of ortho-halogenated phenols (Ref. 10, Table II).

**FIG. 5.** EPR spectra of D. dehalogenans o-CP dehalogenase. Trace A, the [4Fe-4S] signal from enzyme fully reduced by illumination with visible light for 50 min in the presence of 20 μM deazaflavin and 2 mM EDTA. Base-on Cob(II)alamin can be detected as a minor component in trace A. Trace B, the base-off Cob(II)alamin signal from enzyme reoxidized by 0.5-min anaerobic incubation with 2 mM Cl-OHPA. Trace C, the [3Fe-4S] signal from enzyme fully oxidized by anaerobic incubation with 2 mM potassium ferricyanide for 5 min. EPR conditions were as follows: microwave frequency, 9.41 GHz; microwave power 5 milliwatts (as follows: microwave frequency, 9.41 GHz; microwave power 5 milliwatts); modulation frequency, 100 kHz; modulation amplitude, 0.63 millitesla; temperature, 9.5 K (trace A), 30 K (trace B), 15 K (trace C).
The role of cobalamin in the reductive dehalogenases from chlorophenol and PCE-degrading organisms is of special interest, since it does not mediate the "usual rearrangement" or alkyl transfer but an elimination reaction (31). Two models have been proposed for the reaction mechanism of PCE dehalogenase. One model involves the formation of a Co(III)–chloroethene carbon–metal bond (2), whereas the second model postulates the formation of a chloroethene radical (4). However, neither of these intermediates has been demonstrated unequivocally for PCE reductive dehalogenases. Based on our data, it is not possible to determine which model applies for ortho-chlorophenol reductive dehalogenase from D. dehalogenans. On one hand, an essential intermediate in the first model, Cob(III)alamin, was not formed upon oxidation of the enzyme. On the other hand, there was no radical formation upon the addition of substrate to the reduced enzyme. The latter could be due to the slow reaction time, which makes it difficult to detect a reactive compound such as a phenol radical. Additional experiments are required in which the supposed radical would be stabilized.

The similarities between the o-chlorophenol reductive dehalogenase of D. dehalogenans and the PCE reductive dehalogenases of Dehalospirillum multivorans and Dehalobacter restrictus on both mechanistic and structural properties as well as their primary sequences suggest that these enzymes constitute a novel class of corrinoid-containing reductases.

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