Purification and Characterization of Tetrachloroethene Reductive Dehalogenase from Dehalospirillum multivorans*

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Tetrachloroethene reductive dehalogenase from the tetrachloroethene-utilizing anaerobe, Dehalospirillum multivorans, was purified approximately 100-fold to apparent homogeneity. The purified dehalogenase catalyzed the reductive dechlorination of tetrachloroethene (PCE) to trichloroethene and of trichloroethene to cis-1,2-dichloroethene with reduced methyl viologen as the electron donor at a specific activity of 2.6 microkat/ml mg. The apparent $K_m$ values for tetrachloroethene and trichloroethene were 0.20 and 0.24 mM, respectively. The apparent molecular mass of the native enzyme was determined by gel filtration to be 58 kDa. Sodium dodecyl sulfate-gel electrophoresis revealed a single protein band with a molecular mass of 57 kDa. One mol of dehalogenase contained 1.0 mol of corrinoid, 9.8 mol of iron, and 8.0 mol of acid-labile sulfur. The pH optimum was about 8.0. The enzyme had a temperature optimum of about 30°C. It was slightly oxygen-sensitive and was thermostable above 50°C. The dechlorination of PCE was stimulated by ammonium ions. Chlorinated methanes severely inhibited PCE dehalogenase activity.

Dehalospirillum multivorans is a strictly anaerobic, Gram-negative bacterium, which is able to grow with tetrachloroethene (PCE) as terminal electron acceptor for the oxidation of different electron donors (1, 2). The bacterium reduces tetrachloroethene via trichloroethene (TCE) to cis-1,2-dichloroethene in the reductive part of its catabolism. The organism is able to grow at the expense of H$_2$ with PCE as sole energy source. Since H$_2$ oxidation cannot be coupled to ATP synthesis via substrate level phosphorylation, the reductive dechlorination of PCE has to be the energy-generating process in this organism. For mechanistic reasons, ATP synthesis coupled to PCE dechlorination has to proceed via a chemiosmotic mechanism. Therefore, this process was referred to as tetrachloroethene respiration (2, 3). In our laboratory, ongoing studies address tetrachloroethene respiration and the various enzymes involved.

The enzyme tetrachloroethene reductive dehalogenase (PCE dehalogenase) catalyzes in vitro the reductive dechlorination of tetrachloroethene and trichloroethene with reduced methyl viologen as the artificial electron donor at catabolic rates (4). All other artificial or physiological electron donors tested were ineffective (4). In cell extracts, the enzyme (exclusively in its reduced state) was light-reversibly inactivated by propyl iodide. This finding was taken as indirect evidence for the involvement of a corrinoid (vitamin B$_{12}$) in the reductive dechlorination of tetrachloroethene and trichloroethene (4). This finding represents a completely new type of biochemical reaction, in which the cobalt of the vitamin B$_{12}$ appears to be subjected to a change in its redox state in the course of the dehalogenation (4). Purification and characterization of the enzyme were performed here to confirm the presence of a corrinoid and to obtain more information about the composition of the enzyme. To our knowledge, this is the first report on the purification of an enzyme mediating the reductive dehalogenation of a chlorinated aliphatic compound.

MATERIALS AND METHODS

Tetrachloroethene was purchased from Merck (Darmstadt, Germany). Trichloroethene was from Ferak (Berlin, Germany), gases (CO$_2$ grade 4.8, N$_2$ grade 4.6, and N$_2$H$_4$ (95/5%)) were supplied by Messer Griesheim (Düsseldorf, Germany). Chromatography media and instruments were from Pharmacia (Uppsala, Sweden). All chemicals used were of the highest available purity, and were purchased from Aldrich (Steinheim, Germany), Boehringer (Mannheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), and Sigma (Deisenhofen, Germany). Bio-Rad Protein Assay was from Bio-Rad Laboratories (München, Germany).

Purification of the Tetrachloroethene Dehalogenase

Step I. Preparation of Cell Extracts—Dehalospirillum multivorans was grown anaerobically on a medium containing pyruvate and fumarate as described elsewhere (3). The bacteria were harvested in the late exponential growth phase by centrifugation at 5,000 × g and 4°C for 20 min, frozen in liquid nitrogen, and stored frozen at -20°C. Cell pellets were resuspended in 50 mM Tris-HCl, pH 7.5 (3 ml/g cell pellet), containing 1 mM MgCl$_2$, 10 mg/ml lysozyme, and 1 mg/ml deoxyribonuclease I and 4°C for 60 min at 37°C. Cell debris was removed by anoxic centrifugation for 30 min at 100,000 × g and 4°C. All steps were performed in an anaerobic chamber with N$_2$/H$_2$ (95/5%) as the gas phase.

Step II. Chromatography on Q-Sepharose—The supernatant was passed through a Q-Sepharose HP column (1.6 by 10 cm) pre-equilibrated with basic buffer (0.15 M KCl, pH 7.5, containing 0.1 M ammonium sulfate). The dehalogenase was eluted with a linear gradient of ammonium sulfate concentration from 0 to 1 M KCl in basic buffer at a KCl concentration of approximately 0.15 M.

Step III. Chromatography on Phenyl-Sepharose—Fractions containing dehalogenase were pooled, and 3.2 mM ammonium sulfate in basic buffer was added to a final concentration of 0.4 M. The solution was applied to a Phenyl-Sepharose HR column (1.6 by 10 cm) pre-equilibrated with 0.4 M ammonium sulfate in basic buffer. The enzyme was eluted with a linear gradient from 0.4 to 0 M (NH$_4$)$_2$SO$_4$ in basic buffer at an ammonium sulfate concentration of approximately 70 mM.

Step IV. Chromatography on Superdex$^{	ext{TM}}$ 75 pg—Fractions containing dehalogenase were pooled. The enzyme solution was passed through a Superdex$^{	ext{TM}}$ 75-pg column (1.6 by 60 cm) pre-equilibrated with potassium phosphate buffer (50 mM, pH 7.5) containing 0.5 mM dithiothreitol and 0.15 M KCl.

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The abbreviations used are: PCE, tetrachloroethene (also called perchloroethylene); TCE, trichloroethene.
Tetrachloroethene Dehalogenase

Analytical Methods

PCE and TCE dehalogenase activities were routinely assayed by spectrophotometric determination of the oxidation of reduced methyl viologen with tetrachloroethene or trichloroethene as electron acceptors at 578 nm (with tetrachloroethene or trichloroethene as electron acceptors at 578 nm) in a cuvette with N2 (120 kPa) as the gas phase. Methyl viologen was reduced chemically up to an absorption at 578 nm of 3.5 by the addition of protein. The velocity was calculated by determining the rate of the reaction between an absorption of 3.0 and 2.0 (decrease of absorption).

The kinetic constants of the dehalogenase were measured using the spectrophotometric test system described above by varying the concentrations of PCE or TCE at a methyl viologen concentration of 1.6 mM in the assay or by varying the concentration of methyl viologen at a PCE concentration of 1 mM. The oxidation of methyl viologen was assayed at 700 nm due to its lower extinction coefficient ($\varepsilon_{700} = 2.31 \text{ mm}^{-1} \text{ cm}^{-1}$) at this wavelength. For the data for the enzyme kinetics, the Michaelis-Menten kinetics (assuming substrate inhibition; where indicated), and the kinetic parameters ($K_m$ and $K$) were calculated using a computer program (GraphPad Prism, GraphPad Software, San Diego).

The pH dependence of the dehalogenase was tested by varying the buffer substances in the assay. Tris maleate was used in the range from pH 5.5 to 7.0 and Tris-HCl in the range from pH 7.5 to 10.6.

The protein concentration was determined according to Bradford (6) with the Bio-Rad Protein Assay using bovine serum albumin as the standard and by measuring the absorbance of the peptide bonds at 205 nm (7). The same results were obtained with both methods. Iron was determined as described by Fish (8) using an iron volumetric standard (Aldrich, Steinheim, Germany). Acid-labile sulfide was estimated according to Rabinowitz (9). The cobalt content of the dehalogenase was calculated by spectrophotometric quantitation of the extracted corrinoids using hydroxocobalamin as the standard (10). The determination of the cobalt content was kindly performed by Dr. R. Heddle and J. Koch (University of Marburg, Germany) using atomic absorption spectroscopy.

The molecular mass of the native enzyme was determined by gel filtration on a Superdex™ 200- pg column as described in step IV of the purification procedure. The standard proteins (in kDa) were ferritin (450), catalase (240), aldolase (160), bovine serum albumin (67), ovalbumin (43), chymotrypsinogen (25), and myoglobin (17.8).

RESULTS

Purification of Tetrachloroethene Dehalogenase — The enzyme was purified from D. multivorans grown on pyruvate and fumarate in the presence of yeast extract. The specific activity of the purified PCE dehalogenase was determined with methyl viologen as substrate and the optimum temperature was found to be 25°C. The enzyme was slightly oxygen-sensitive and lost about 50% of its activity during incubation and stirring for 2 h at 4°C in the presence of air. The dehalogenase was judged to be homogeneous on the basis of polyacrylamide gel electrophoresis in the presence of SDS (see Fig. 2).

Protein Composition— Gel filtration on Superdex™ 200-pg column indicated an apparent molecular mass for the active PCE dehalogenase of 58 kDa (Fig. 1). SDS-polyacrylamide gel electrophoresis revealed a single protein band, indicating that the dehalogenase consists of one polypeptide. The apparent molecular mass of the monomeric enzyme was calculated to be 57 kDa (Fig. 2).

The UV-visible spectrum of the enzyme exhibited absorption maxima at 280 and at about 400 nm (Fig. 3). The absorption maximum at 400 nm was probably due to the presence of iron-sulfur clusters (see below). The purified enzyme was light-reversibly inactivated by propyl iodide in the presence of tita-

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The UV-visible spectrum of the enzyme exhibited absorption maxima at 280 and at about 400 nm (Fig. 3). The absorption maximum at 400 nm was probably due to the presence of iron-sulfur clusters (see also Ref. 4). The lack of distinct absorption maxima at near 520 and 550 nm (typical for cob(III)alamin) and at about 385 nm (typical for cob(III)alamin) well as well as the shoulder in the absorption spectrum at about 310 nm (absorption maximum of cob(III)alamin) may be taken to indicate the presence of a corrinoid in the cob(III)alamin form (Fig. 3).

The PCE dehalogenase was incubated for 10 min at 100°C in the presence of cyanide to extract corrinoids present in the enzyme as dicyano-cobalamin. The absorption spectrum of the extract exhibited the maxima typical for dicyano-cob(III)-alamin (Fig. 3, inset). From the absorption difference $A_{385} - A_{550}$ the corrinoid content of the enzyme was estimated to be 1.1 mol of corrinoid/mol of protein using dicyano-cobalamin as the standard (10). The cobalt content of the enzyme was determined by atomic absorption spectroscopy to be 1.0 mol of cobalt/mol of protein (Table I). These data suggested the presence of 1 mol of corrinoid/mol of protein. The iron content of the dehalogenase was determined to be 9.8 mol of iron/mol of protein. The content of acid-labile sulfur was estimated to be 8.0 mol of sulfur/mol of protein (Table I).

Catalytic Properties — The purified PCE dehalogenase exhibited a pH optimum at about 8; the optimal temperature was near 42°C (data not shown). The apparent $K_m$ values for PCE and TCE were determined at a methyl viologen concentration of 1.6 mM at 700 nm to be 0.20 and 0.24 mM, respectively (Fig. 4, A and B). Enzyme inhibition by PCE ($K_I = 18$ mM; Fig. 4A) and TCE ($K_I = 39$ mM; Fig. 4B) was observed. Higher concentrations of methyl viologen could not be applied, since it was not possible to measure initial absorptions higher than 4 in the spectrophotometric enzyme assay. The apparent $K_m$ for methyl viologen was determined as 1 nmol chloride released or 2 nmol methyl viologen oxidized per sec at 25°C.

Table I: Purification of tetrachloroethene dehalogenase from D. multivorans 30 g, wet weight, cells were used in this preparation. For experimental detail, see "Materials and Methods."

| Purification step | Total activity | Yield | Specific activity | Purification factor |
|------------------|---------------|-------|------------------|--------------------|
| Crude extract    | nkat*         | %     | nkat* mg         | -fold              |
| Q-Sepharose      | 31670         | 100   | 25               | 1                  |
| Phenyl-Superose  | 26920         | 85    | 153              | 6                  |
| Superdex™ 75 pg  | 21220         | 67    | 2500             | 100                |
| Superdex™ 200 pg | 20270         | 64    | 2640             | 106                |

* 1 nkat is defined as 1 nmol chloride released or 2 nmol methyl viologen oxidized per sec at 25°C.
viologen was determined at a PCE concentration of 1 mM to be 0.28 mM (Fig. 4C).

Of the halogenated ethenes tested besides PCE and TCE, only tetraiodoethene was reduced by the enzyme; the reaction rate was considerably slower than with PCE (data not shown). 1,1-Dichloroethene, trans-1,2-dichloroethene, 1,1,1-trichloroethene, 1,1,1,2-tetrachloroethene, and chloromethanes are able to serve as substrates for microorganisms. PCE is one of these compounds (1–3). Due to its persistence under oxidative conditions, however, PCE can apparently only be reductively dechlorinated.

The catabolic PCE dehalogenase of D. multivorans mediates in vitro the reductive dechlorination of tetrachloroethene to cis-1,2-dichloroethene with reduced methyl viologen as the artificial electron donor. At present, the physiological electron donor of the dehalogenase is not known (see also Ref. 4). Besides PCE, only TCE and tetraiodoethene are dehalogenated by the enzyme. Neither other chloroethenes nor chloroethanes and chloromethanes are able to serve as substrates for the PCE dehalogenase. This finding, along with the high specific activity in cell extracts and the low apparent Ki value for PCE of the purified enzyme, leads to the conclusion that the PCE dehalogenase is a specific enzyme highly suited for the dechlorination of PCE and TCE. This is surprising, as these compounds are xenobiotics and have been present in the environment in significant concentrations starting about 50 years ago. Therefore, the evolutionary origin of the enzyme is a highly interesting problem, which might be solved after cloning and sequencing the gene. This work is currently under progress in our laboratory.

The tetrachloroethene dehalogenase consists of a single polypeptide with a molecular mass of 57 kDa. A corrinoid was extracted from the enzyme as dicyano-cobalamin (due to the extraction conditions) in a ratio of 1 mol of corrinoid/mol of enzyme. Besides a corrinoid, iron-sulfur clusters are present as additional cofactors. The only other reductive dehalogenase purified so far is the 3-chlorobenzoate dehalogenase of Desulfovomile tiedjei (13). This enzyme exhibits properties completely different from those of the PCE dehalogenase. The

**DISCUSSION**

Carbon-halogen bond cleavage is a critical step in the microbial utilization of halogenated compounds. Different reaction mechanisms were described including hydrolytic, oxidative, and reductive dehalogenation (see Refs. 11, 12 for an overview). Few halogenated compounds may serve as growth-supportive substrates for microorganisms. PCE is one of these compounds (1–3). Due to its persistence under oxidative conditions, however, PCE can apparently only be reductively dechlorinated.

The iron, sulfur, corrinoid, and cobalt content of tetrachloroethene dehalogenase

| Sample | Protein | Protein | Iron | Sulfur | Corrinoid | Cobalt |
|--------|---------|---------|------|--------|-----------|--------|
|        | mg/ml   | μM      | μM   | μM     | μM        | μM     |
| 1      | 0.52    | 9.1     | 70   | 57.0   | ND a      | ND a   |
| 2      | 0.39    | 6.7     | ND a | 58.5   | 7.5       | ND a   |
| 3      | 0.35    | 6.1     | 65   | 54.5   | ND a      | 6.0    |
|        |         |         | 67   |        |           |        |
| Average stoichiometry | 9.8 | 8.0 | 1.1 | 1.0 |

a ND, not determined.
3-chlorobenzoate dehalogenase consists of 2 subunits (αβ-structure) with molecular masses of 64 and 37 kDa. The absorption spectrum was interpreted by the authors to indicate the presence of 1 mol of heme per mol of protein (possibly a c-type cytochrome). Other iron-containing cofactors or corrinoids were not detected (13). Therefore, it is concluded that the dehalogenases are different enzymes and most probably involved different dehalogenation mechanisms. It should be noted, however, that the specific activity of the 3-chlorobenzoate reductive dehalogenase (13) was by several orders of magnitude lower than that of the PCE dehalogenase, thus impeding studies on the composition and reaction mechanism of 3-chlorobenzoate dehalogenase. The PCE dehalogenase was obtained at high yield and high specific activity as is typical for catabolic enzymes.

Earlier work (4) and the present study point to the crucial role of vitamin B12 in the reaction mechanism. Propyl iodide inactivated the enzyme exclusively after reduction of the enzyme by titanium(III) citrate. This indicates that the corrinoid in the cob(I)alamin state is alkylated by propyl iodide. It thus is likely that tetrachloroethene also serves as an alkylating agent for the corrinoid in the reaction. Evidence was presented that the cobalt in the enzyme as isolated is probably present in the 2⁺ oxidation state. This means that the cobalt could undergo a redox change in the course of the dehalogenation. The inhibition of the PCE dehalogenase by cyanide was interpreted to indicate the involvement of cob(III)alamin in the reaction (4). For these reasons, we favor a reaction mechanism, in which the corrinoid is reduced to the cob(I)alamin prior to alkylation with PCE. The alkyl residue could then be protonolytically eliminated, depriving the cobalt of its σ-bond electrons; cob(III)alamin may thus be formed. Then cob(III)alamin has to be reduced again to cob(I)alamin. Whether this is performed in a single two-electron transfer step or in two one-electron transfer reactions is not yet known. For the reduction of cob(III)alamin to cob(I)alamin, an electron at a relatively positive redox potential would be sufficient, whereas an electron with a negative redox potential is required for the reduction of Co(II) to Co(I) in the corrinoid. (In the corrinoid/iron-sulfur protein of Clostridium thermoaceticum for example, $E^\circ_{Co(III)alamin/Co(II)alamin} = +200 \text{mV}$ and $E^\circ_{cob(II)alamin/cob(I)alamin} = −520 \text{mV}$ (14).) Therefore, a “splitting” of the two reducing equivalents for the reductive dechlorination of PCE into a low redox potential and a high redox potential electron is feasible. In this case, the presence of two different electron-transferring cofactors in the enzyme would probably be necessary. The iron-sulfur content of the enzyme allows for two Fe₄S₄ clusters in the dehalogenase. This is in accordance with the working hypothesis described above and depicted in the tentative scheme given in Fig. 6. An example for such an electron splitting is the ubiquinol oxidase from Escherichia coli (15). The authors (15) postulate that the high affinity quinone-binding site of the ubiquinol oxidase “functions as a molecular gate which regulates electron flux from the two-electron redox component to one-electron carriers.”

A splitting of the reducing equivalents would also make sense in light of the mechanism of energy conservation that is coupled to tetrachloroethene reductive dechlorination. Our previous studies on the cellular localization of the enzymes involved in tetrachloroethene respiration indicated the presence of a cytoplasmic PCE dehalogenase (1) and a membrane-associated, periplasmic hydrogenase. Low potential electrons are required in any case in order to provide cob(I)alamin for the

\[ \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} \]

}\[ \text{Fe}^{3+} \rightarrow \text{Fe}^{4+} \]

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2 E. Miller, G. Wohlfarth, and G. Diekert, unpublished data.
alkylation. These low potential electrons have to be translocated from the hydrogenase outside the cell (E$_{0}$ of the H$^+$/H$_2$ couple = $-414$ mV) to the PCE dehalogenase inside the cell (E$_{0}$ of cob(II)alamin/cob(I)alamin estimated to be $-0.4$ to $-0.5$ V) across the cytoplasmic membrane. Assuming a membrane potential of about $-200$ mV (outside positive), which is a value typical for bacterial cells (16), the electron translocation must be endergonic. Therefore, a two-electron transfer is not feasible. The thermodynamic problem could be circumvented, however, if the reducing equivalents are “split” with respect to their redox potential. Were this the case, one electron could be transferred from the hydrogenase to a positive redox potential electron acceptor inside the cell for the reduction of cob(III)alamin to cob(II)alamin in an exergonic reaction. This reaction could drive the thermodynamically unfavorable electron transfer from the hydrogenase to a low potential electron carrier inside the cell for the reduction of cob(II)alamin to cob(I)alamin (Fig. 6). Therefore, the reaction mechanism of the PCE dehalogenase may be closely linked to the mechanism of tetrachloroethene respiration and the electron transfer reactions involved. Studies on the redox potentials of the prosthetic groups of PCE dehalogenase (B$_{12}$ and Fe-S clusters) will help to unravel both the reaction mechanism of the enzyme and the electron transport. These studies are currently under way in our laboratory.

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