Purification and Characterization of a Novel 3-Chlorobenzoate-Reductive Dehalogenase from the Cytoplasmic Membrane of Desulfomonile tiedjei DCB-1

SHUISONG NI, JAMES K. FREDRICKSON, AND LUYING XUN

Environmental Microbiology Group, Pacific Northwest Laboratory, and Department of Microbiology, Washington State University Tri-Cities, Richland, Washington 99352

Received 15 February 1995/Accepted 4 July 1995

Although reductive dehalogenation by anaerobic microorganisms offers great potential for the degradation of halocarbons, little is known about the biochemical mechanisms involved. It has previously been demonstrated that the dehalogenase activity involved in 3-chlorobenzoate dehalogenation by Desulfomonile tiedjei DCB-1 is present in the membrane fraction of the cell extracts. We report herein the purification of a 3-chlorobenzoate-reductive dehalogenase from the cytoplasmic membrane of D. tiedjei DCB-1. The dehalogenase activity was monitored by the conversion of 3-chlorobenzoate to benzoate with reduced methyl viologen as a reducing agent. The membrane fraction of the cell extracts was obtained by ultracentrifugation, and the membrane proteins were solubilized with either the detergent CHAPS (3-[3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate) or Triton X-100 in the presence of glycerol. The solubilized dehalogenase was purified by ammonium sulfate fractionation and a combination of anion exchange, hydroxyapatite, and hydrophobic interaction chromatographies. This procedure yielded about 7% of the total dehalogenase activity with a 120-fold increase in specific activity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the purified dehalogenase consisted of two subunits with molecular weights of 64,000 and 37,000. The enzyme converted 3-chlorobenzoate to benzoate at its highest specific activity in 10 mM potassium phosphate buffer (pH 7.2) at 38°C. The enzyme was yellow and probably a heme protein. The enzyme had an absorbance peak at 408 nm. The dithionite-reduced enzyme displayed absorbance peaks at 416, 522, and 550 nm. The dithionite-reduced enzyme was able to grow on a variety of compounds including pyruvate, succinate, formate, vanillate, and H2-CO2(8,15). Strain DCB-1 can use sulfate, sulfite, or thiosulfate as an electron acceptor (20,27).

MATERIALS AND METHODS

Organism and growth conditions. D. tiedjei DCB-1 was obtained from Todd Stevens and William W. Mohn. The modified (18) anaerobic techniques of Hungate (13) were used for the cultivation of strain DCB-1. The organism was routinely grown in the medium previously reported (8) with some modifications. Our medium contained the following (per liter): NH4Cl, 1.00 g; KCl, 0.10 g; KH2PO4, 0.10 g; MgSO4, 7H2O, 0.17 g; CaCl2, 0.02 g; NaCl, 1.17 g; NaHCO3, 3.00 g; K2(PIPES) [piperazine-N,N'-bis-(2-ethanesulfonic acid)], 1.50

Copyright © 1995, American Society for Microbiology
g: yeast extract (Difco, Detroit, Mich.), 2.00 g: treptone (Difco), 2.00 g: trace minerals (2), 10.00 ml: vitamin mixture (2, 8), 1.00 ml: resazurin, 1.00 mg: 2.00% 10,000 times. The medium was prepared by adding all the components from concentrated stock solutions in O2-free distilled water and equilibrating the preparation with N2-CO2 (4:1). The pH of the medium was around 7.0. After it was autoclaved but before it was inoculated, the medium was reduced by adding titanium citrate (33) to 0.1 mM and supplemented with pyruvate to 40 mM and 3-chlorobenzoate to 0.2 mM. The culture was incubated at 37 °C and 100% humidity for 2 hours to aerate the sample. After 2 hours, the dehalogenase activity could be detected in the absence of either methyl viologen or H2. The dehalogenase was an inducible membrane protein. Cellextracts prepared from cultures grown on pyruvate without 3-chlorobenzoate did not produce any benzene from 3-chlorobenzoate in the complete assay mixture. After ultrafiltration at 200,000 × g for 2 hours, the dehalogenase activity was present exclusively in the sediment containing the particulate membrane fraction. Because exposure of the dehalogenase to air during cell collection and cell disruption by French press did not inactivate its dehalogenating activity when the buffer contained 1 mM dithiothreitol, purification procedures were carried out under aerobic conditions.

**Solubilization of the dehalogenase.** The ultracentrifuged membrane fraction was fractionated into three phases by 55% saturation of ammonium sulfate after centrifugation (protein precipitate at the bottom, Triton X-100 layer floating on the top, and soluble proteins in between). About 80% of the dehalogenase activity was recovered in the floating Triton X-100 layer (Table 1). The specific activity of the dehalogenase increased twofold at this stage. The membrane proteins were solubilized by suspending the floating Triton X-100 layer in buffer III. After dialysis and

**Analytical methods.** Benzoate and 3-chlorobenzoate were analyzed with an HPLC apparatus (Waters, Millford, Mass.) equipped with a Waters 960 photo-diode array detector. Samples were mixed with acetonitrile (1:1) and centrifuged at 13,000 g for 3 minutes to remove precipitated proteins. The supernatant (10 μl) was injected onto a Nova-pak C18 column (3.9 by 150 mm; pore size, 60 Å [6 μm]) equilibrated with 11 mM H3PO4 containing 20% acetonitrile. The column was then eluted at a flow rate of 1.0 ml min−1 with an 11 mM H3PO4 acetonitrile gradient (acetonitrile concentrations: 20 to 70% [linear, 5 min], 70% [isocratic, 60% [isocratic, 1 min], and 100 to 20% [linear, 1 min]) (31). A240 to A450 were monitored. Benzoate and 3-chlorobenzoate were distinguished by their respective retention times of 5.8 and 7.6 and absorption peaks at 260 and 284 nm. Benzoate and 3-chlorobenzoate were quantified by comparing their peak areas with those of standards.

To analyze protein purity and estimate protein molecular weight, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) assays were carried out in 12% ready gels (Bio-Rad) by the method of Laemmli (14). Protein concentrations were measured with the micro bichoninic acid protein assay kit (Pierce, Rockford, Ill.). Samples were dialyzed to remove dithiothreitol, which could otherwise interfere with the bicinchoninic acid method.

UV-visible spectra of the purified dehalogenase were obtained with a Perkin-Elmer Lambda 9 spectrophotometer. The dehalogenase used in this experiment was inactive because of the precipitation of the protein during the process of concentrating the sample. Absorbance of the dehalogenase sample (15 μl at 6.4 mg ml−1) in 50 mM potassium phosphate (pH 7.2) containing CHAPS and 20% glycerol was measured at between 350 and 700 nm in a glass melting-point capillary tube (∼1.2 to 1.5 mm; inside diameter; Kimax). The protein was then reduced by adding sodium dithionite (0.1 mM) to an Ar-purged sample. After the spectrum of the dithionite-reduced enzyme was recorded, the solution was gassed with a steady flow of pure carbon monoxide (CO) for the spectrum of the CO-complexed enzyme. All the spectra were analyzed and processed with LabCalc software (Gálactic Industries, Salem, N.H.).

For the reverse-phase HPLC, the protein (50 μg) was loaded onto a Microcolumn (M) C8 300-Å (30 mm)-pore-size column (Rainin Instrument Co., Inc., Emeryville, Calif.) equilibrated with 30% acetonitrile containing 0.1% trifluoroacetic acid. The column was eluted at a flow rate of 1 ml min−1 with a water-acetonitrile gradient containing 0.1% trifluoroacetic acid (acetonitrile concentrations: 30 to 50% [linear, 0.5 min], 50 to 100% [linear, 9.5 min], 100% [isocratic, 5 min], and 100 to 30% [linear, 1 min]). Absorption was monitored between 250 and 500 nm. As absorbance peaks appeared, the eluants were collected for SDS-PAGE analysis.

**RESULTS**

**Dehalogenase activity in cell extracts.** Cell extracts (10 mg ml−1) of DCB-1 converted 0.5 mM 3-chlorobenzoate completely to benzene after 10 hours of incubation at 37°C in the presence of methyl viologen and H2. Methyl viologen was reduced within 10 min of incubation by the indigenous hydrogenase from the cell extracts. The conversion was enzymatic, because formation of benzene was not detected in controls without cell extracts or with boiled cell extracts. No dehalogenase activity was detected in the absence of either methyl viologen or H2. The dehalogenase was an inducible membrane protein. Cell extracts prepared from cultures grown on pyruvate without 3-chlorobenzoate did not produce any benzene from 3-chlorobenzoate in the complete assay mixture. After ultrafiltration at 200,000 × g for 2 hours, the dehalogenase activity was present exclusively in the sediment containing the particulate membrane fraction. Because exposure of the dehalogenase to air during cell collection and cell disruption by French press did not inactivate its dehalogenating activity when the buffer contained 1 mM dithiothreitol, purification procedures were carried out under aerobic conditions.

**Dehalogenase assays.** The dehalogenase activity in the cell extracts was assayed in 50 μl of 50 mM potassium phosphate buffer (pH 7.8) containing 0.5 mM 3-chlorobenzoate, 0.1 mM methyl viologen, and 0.1 mM sodium dithionite. The sample (with the protein concentration ranging from 0.5 to 10 mg ml−1) in a high-performance liquid chromatography (HPLC) sampling vial, which was then placed in a Hugate tube. The headspace of the Hugate tube was filled with 100% H2, and the tubes were sealed with butyl rubber stoppers and screw caps. The mixture was incubated at 37°C for 8 to 10 hours. During purification, the assay mixture was modified to include 20% glycerol and 6 mM CHAPS (or 1% Triton X-100). About 5 μg of a hydrogenase, which was partially purified from DCB-1 (data not shown), was added to the assay mixture to reduce methyl viologen. The activity of the purified enzyme was also tested in a similar assay mixture, except that the hydrogenase and H2 were replaced by 50 mM sodium citrate (33) and O2.

The temperature optimum of the dehalogenase was determined in 50 mM phosphate buffer (pH 7.8) at between 20 and 65°C at 5°C intervals. For optimal pH, the enzyme was tested at different pH values between 6.0 and 9.0 in either 50 mM potassium phosphate buffer or 50 mM Tris buffer at 38°C. The dehalogenase activity was dependent on the pH of the reaction mixture.

The dehalogenase activity was assayed at different pH values between 6.0 and 9.0 in 50 mM potassium phosphate buffer or 50 mM Tris buffer at 38°C. The dehalogenase activity was dependent on the pH of the reaction mixture.

The dehalogenase activity was assayed at different pH values between 6.0 and 9.0 in 50 mM potassium phosphate buffer or 50 mM Tris buffer at 38°C. The dehalogenase activity was dependent on the pH of the reaction mixture.

The dehalogenase activity was assayed at different pH values between 6.0 and 9.0 in 50 mM potassium phosphate buffer or 50 mM Tris buffer at 38°C. The dehalogenase activity was dependent on the pH of the reaction mixture.
TABLE 1. Purification scheme for the 3-chlorobenzoate-reductive dehalogenase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol (ml)</th>
<th>Protein (mg)</th>
<th>Activity (U)*</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>30</td>
<td>450</td>
<td>68.7</td>
<td>0.15</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation</td>
<td>20</td>
<td>160</td>
<td>52.1</td>
<td>0.33</td>
</tr>
<tr>
<td>Membrane protein extract</td>
<td>20</td>
<td>76</td>
<td>34.8</td>
<td>0.46</td>
</tr>
<tr>
<td>High Q</td>
<td>4.5</td>
<td>4.5</td>
<td>21.9</td>
<td>4.87</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>2.5</td>
<td>0.88</td>
<td>9.3</td>
<td>10.63</td>
</tr>
<tr>
<td>Octyl agarose</td>
<td>1.3</td>
<td>0.26</td>
<td>4.8</td>
<td>18.46</td>
</tr>
</tbody>
</table>

* One unit is defined as the amount of protein which catalyzed the formation of 1 nmol of benzoate per min in the assay.

ultracentrifugation, about 50% of the dehalogenase activity was present in the supenatant (Table 1). Inclusion of 20% glycerol in the buffer was essential for solubilizing the dehalogenase from the membrane. In the absence of glycerol, 3% Triton X-100 (about 1:1 [wt/wt] detergent-to-protein ratio) was required for its solubilization. The dehalogenase activity, however, was not stable in buffers with Triton X-100 concentrations above 3%. When Triton X-100 in buffer III was replaced with 6 mM CHAPS, only 30% of the total dehalogenase activity was solubilized.

**Purification of the dehalogenase.** The solubilized dehalogenase was purified by a combination of anion exchange, hydroxyapatite, and hydrophobic interaction chromatographies. The dehalogenase from the High Q anion-exchange column was eluted with the starting buffer (buffer IV), and the second activity peak was eluted with about 0.6 M NaCl. After the sample was loaded onto the High Q column preequilibrated with buffer IV containing CHAPS, some of the dehalogenase initially solubilized in Triton X-100 may be partitioned into zwitterionic CHAPS micelles and bound to the column. The rest of the dehalogenase, which still remained in nonionic Triton X-100 micelles, did not bind to the column and passed through the column. The first peak contained about two-thirds of the total activity applied to the column, and the specific activity of the dehalogenase increased 10-fold. Two dominant bands together with other minor protein bands were revealed by SDS-PAGE. These two bands were consistently present in the fractions with dehalogenase activity during the rest of the purification process. The two bands were also present but were not dominant when the second activity peak was analyzed by SDS-PAGE.

Fractions within the first activity peak were pooled and loaded onto the hydroxyapatite column. The dehalogenase was eluted in a single peak with 150 to 200 mM potassium phosphate. A purification of about twofold was achieved by this step. Another twofold purification was accomplished by hydrophobic interaction chromatography on an octyl agarose column. The dehalogenase bound strongly to this column in buffer V containing a 20% saturation of ammonium sulfate. After the column was washed with buffer IV, the dehalogenase was eluted with buffer VI containing 1% Triton X-100. When the fractions associated with the second activity peak from the High Q column were purified by the same methods, the resulting dehalogenase was identical to that from the first High Q activity peak, but with more impurities.

This overall procedure yielded about 7% of the dehalogenase activity initially present in the cell extracts with a 120-fold increase in its specific activity (Table 1). SDS-PAGE analysis (Fig. 1) revealed two bands with equal densities and few impurities. The molecular weights of these two bands were estimated to be 64,000 and 37,000. The purified enzyme was active when it was assayed with titanium-reduced methyl viologen. This indicated that the partially purified hydrogenase in our routine assay mixture only provided the reduced methyl viologen and did not have additional roles in the dehalogenation of 3-chlorobenzoate.

Figure 2 shows a typical time course for the formation of benzoate from 3-chlorobenzoate by the dehalogenase. The pure enzyme exhibited its highest specific activity at 38°C between pH 7.2 and 7.5 and at the ionic strength equivalent to 10 mM potassium phosphate. The dehalogenase was active over temperatures ranging from 20 to 55°C, pHs from 6.5 to 8.7, and potassium phosphate concentrations from 10 to 300 mM. It was completely inactivated above 60°C, at pHs above 9, or at 400 mM potassium phosphate. The pure dehalogenase tended to be precipitated by being concentrated or frozen and thawed, and the precipitated enzyme lost its activity completely.

**Spectral properties of the dehalogenase.** As can be seen from Fig. 3, the oxidized dehalogenase displayed a maximum absorbance at 408 nm. When the enzyme was reduced by dithionite under anaerobic conditions, the peak shifted to 416 nm. The reduced enzyme also displayed two additional peaks at 550 and 522 nm, resembling the α and β peaks of heme proteins. When the dithionite-reduced protein was exposed to CO, the peak at 416 nm shifted back to 413 nm, indicating that CO could bind to the dithionite-reduced enzyme. The absorbance peaks between 460 and 500 nm seen in all the spectra were artifacts due to the use of the glass capillary tube. In another experiment in which the sample had a low concentra-

FIG. 1. SDS-PAGE gel with the purified 3-chlorobenzoate-reductive dehalogenase (1.4 μg; lane B). Molecular size markers are shown in lane A. The numbers at the left are kilodaltons.

FIG. 2. A typical time course for the formation of benzoate from 3-chlorobenzoate by the dehalogenase. The pure enzyme exhibited its highest specific activity at 38°C between pH 7.2 and 7.5 and at the ionic strength equivalent to 10 mM potassium phosphate. The dehalogenase was active over temperatures ranging from 20 to 55°C, pHs from 6.5 to 8.7, and potassium phosphate concentrations from 10 to 300 mM. It was completely inactivated above 60°C, at pHs above 9, or at 400 mM potassium phosphate. The pure dehalogenase tended to be precipitated by being concentrated or frozen and thawed, and the precipitated enzyme lost its activity completely.
tion (<0.5 mg ml⁻¹) of the purified active dehalogenase, no absorbance peaks between 500 and 650 nm were observed. The yellow chromophore could not be separated from the protein after the enzyme was denatured with 5% trichloroacetic acid. However, the tight binding of the chromophore to the protein allowed us to identify the subunit associated with the chromophore. The two subunits of the dehalogenase were separated by reverse HPLC without losing the chromophore. As expected, the purified dehalogenase had two major protein peaks with retention times of 5.8 and 7.4 min. The protein peak at 7.4 min had two absorbance maxima at 280 and 396 nm (Fig. 4). This strongly suggested that the yellow chromophore was associated with the second protein peak at 7.4 min. The absorbance peak of the chromophore shifted from 409 nm probably because of the acidic conditions of the elution buffer. SDS-PAGE analyses of the eluants from the reverse-phase HPLC column showed that the second protein peak at 7.4 min contained the smaller subunit of the dehalogenase.

**DISCUSSION**

These results describe the first reductive dehalogenase purified from an anaerobic dehalogenating bacterium. The enzyme was nearly homogeneous after the 120-fold purification procedure. It contained two bands of similar densities, as shown by SDS-PAGE analysis. We speculate that the native dehalogenase consisted of two subunits. It is less likely that these two bands represented two separate enzymes that happened to be copurified on all the columns we used. However, the hydrophobic nature of the protein makes it unsuitable for further analysis by either native PAGE or gel filtration chromatography. This enzyme was distinct from the *Flavobacterium* tetrachloro-p-hydroquinone-reductive dehalogenase that is a soluble protein using glutathione as its electron donor (32). The 3-chlorobenzoate reductive dehalogenase is likely to be an integral membrane protein that is involved in the energy metabolism of strain DCB-1 (10, 21, 22).

The 3-chlorobenzoate reductive dehalogenase is hypothesized to be the terminal reductase of an uncharacterized electron transport chain (22, 23). It is essential to use an artificial reducing agent to replace the native electron donor for the monitoring of dehalogenase activity during purification. As was previously shown, reduced methyl viologen can provide reducing power for the dehalogenation of 3-chlorobenzoate in cell extracts of *D. tiedjei* (9). In the absence of methyl viologen, the rate of 3-chlorobenzoate dehalogenation is much slower than that when methyl viologen is present (9). When we omitted methyl viologen from the assay mixture, dehalogenase activity was not detected in the cell extracts. It is possible that the native electron transport chain consists of electron carriers located in both the membrane and the cytosol and that disruption of the electron transport chain during cell breakage caused partial or complete loss of the dehalogenase activity in the cell extracts. Reduced methyl viologen turned out to be effective as the reducing agent throughout the purification process. These results indicate that reduced methyl viologen can circumvent the entire electron transport chain and directly reduce the redox center of the reductive dehalogenase. The reduced enzyme, in turn, converts 3-chlorobenzoate to benzoate.

The yellow chromophore associated with the smaller subunit might be the redox center for the dehalogenase. Binding of CO to the dithionite-reduced dehalogenase was indicative of the presence of a metalloporphyrin. A preliminary analysis of the dehalogenase by atomic absorbance spectroscopy showed that the enzyme contained Fe (data not shown). The UV-visible spectra indicated that the dehalogenase was a heme protein. The α peak at 550 nm of the dithionite-reduced dehalogenase resembled those of c-type cytochromes (4). However, the nature of the heme is currently unknown. A recent study of the respiratory components of *D. tiedjei* DCB-1 also showed the possible involvement of a c-type cytochrome in reductive dehalogenation (16). Many abiotic systems containing hemes (23) and several heme proteins including bacterial cytochrome P-450 (5) and c-type cytochromes from *Shewanella putrefaciens* 200 (25) are also capable of reductive dehalogenation, but most of their activities are nonspecific and limited only to the degradation of halogenated alkyl compounds. The 3-chloro-

**FIG. 3.** UV-visible spectra of the oxidized (a), dithionite-reduced (b), and CO-complexed (c) 3-chlorobenzoate-reductive dehalogenase.

**FIG. 4.** Reverse-phase HPLC chromatogram (A) of 3-chlorobenzoate-reductive dehalogenase monitored at 400 nm and the index spectrum (B) of the absorbance peak at 7.4 min.
benzoate-reductive dehalogenase of *D. tiedjei* may contain a unique redox center for aryl-reductive dehalogenation.

**ACKNOWLEDGMENTS**

We thank Todd Stevens (Pacific Northwest Laboratory) and William W. Mohn (University of British Columbia, Vancouver, British Columbia, Canada) for providing us with cultures of *D. tiedjei* DCB-1. We are also grateful to Jie Sun of the Oregon Graduate Institute for the UV-visible spectroscopic analysis.

This work was supported by the Microbial Biotechnology Initiative at the Pacific Northwest Laboratory. The Pacific Northwest Laboratory is operated for the U.S. Department of Energy by the Battelle Memorial Institute under contract DE-AC06-76RLO 1830.

**REFERENCES**


ments and substrates for reductive dehalogenation by strain DCB-1. J. Ind. Microbiol. 5:9–16.


