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Purification and characterization of the tetrachloroethene reductive dehalogenase of strain PCE-S

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Abstract The membrane-associated tetrachloroethene reductive dehalogenase from the tetrachloroethene-reducing anaerobe, strain PCE-S, was purified 165-fold to apparent homogeneity in the presence of the detergent Triton X-100. The purified dehalogenase catalyzed the reductive dechlorination of tetrachloroethene to trichloroethene and of trichloroethene to *cis*-1,2-dichloroethene with reduced methyl viologen as the electron donor, showing a specific activity of 650 nkat/mg protein. The apparent K_m values of the enzyme for tetrachloroethene, trichloroethene, and methyl viologen were 10 µM, 4 µM, and 0.3 mM, respectively. SDS-PAGE revealed a single protein band with an apparent molecular mass of 65 kDa. The apparent molecular mass of the native enzyme was 200 kDa as determined by gel filtration. Tetrachloroethene dehalogenase contained 0.7 ± 0.3 mol corrinoid, 1.0 ± 0.3 mol cobalt, 7.8 ± 0.5 mol iron, and 10.3 ± 2.0 mol acid-labile sulfur per mol subunit. The pH optimum was approximately 7.2, and the temperature optimum was approximately 50 °C. The dehalogenase was oxygen-sensitive with a half-life of approximately 50 min. The N-terminal amino acid sequence of the enzyme was determined, and no significant similarity was found to any part of the amino acid sequence of the tetrachloroethene (PCE) reductive dehalogenase from *Dehalospirillum multivorans*.

Key words Corrinoid protein · *Desulfitobacterium* · Iron-sulfur protein · N-terminal amino acid sequence · PCE dehalogenase · Strain PCE-S · Tetrachloroethene reductive dehalogenase

Abbreviations *PCE* Tetrachloroethene · *TCE* Trichloroethene

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Introduction

Members of the gram-positive genus *Desulfitobacterium* are able to reductively dehalogenate a variety of chlorinated hydrocarbons including chlorinated phenols and/or alkenes in their energy metabolism. To date, only two strains of the genus *Desulfitobacterium*, namely strain PCE1 (Gerritse et al. 1996) and strain PCE-S (Miller et al. 1997 b), are known to dechlorinate tetrachloroethene reductively. In addition, enzyme-catalyzed tetrachloroethene reductive dechlorination in pure cultures has been described for the gram-negative bacteria *Dehalospirillum multivorans* (Neumann et al. 1994, 1995; Scholz-Muramatsu et al. 1995; Miller et al. 1997 a), *Desulfomonile tiedjei* (Fathepure et al. 1987), strain MS-1 (Sharma and McCarty 1996), and strain TT4B (Krumholz et al. 1996) as well as for the gram-positive bacteria *"Dehalobacter restrictus"* (Holliger and Schumacher 1994; Schumacher and Holliger 1996) and the phylogenetically unrelated *Dehalococcoides ethenogenes* strain 195 (Maymó-Gatell et al. 1997). For recent reviews on microbial reductive dehalogenation, the reader is referred to Wohlfarth and Diekert (1997) and Holliger et al. (1997).

Strain PCE-S is a strictly anaerobic, gram-positive bacterium that is able to utilize various electron acceptors in its energy metabolism such as fumarate, tetrachloroethene, and trichloroethene. Strain PCE-S will probably be designated as *Desulfitobacterium frappieri* strain PCE-S in a separate manuscript (S. Granzow, H. Scholz-Muramatsu, C. Strömpel, E. Moore and G. Diekert; unpublished work). The bacterium reduces tetrachloroethene via trichloroethene to *cis*-1,2-dichloroethene in the reductive part of its catabolism. Cell extracts of pyruvate- and fumarate-grown cells catalyze the reductive dechlorination of tetrachloroethene and trichloroethene exclusively with low-potential reduced viologens (e.g., methyl viologen, triquat) as artificial electron donors at catabolic rates (Miller et al. 1997 b). Chlorinated aromatic compounds are not converted under the conditions applied. In cell extracts, the tetrachloroethene (PCE) dehalogenase activity is light-re-

versibly inactivated by propyl iodide in its reduced state, indicating the involvement of a corrinoid in the dehalogenation reaction. Up to 30% of the enzymatic activity is found in the membrane fraction of strain PCE-S, indicating a membrane association of the PCE dehalogenase (Miller et al. 1997 b).

Recently, for the first time a PCE reductive dehalogenase has been purified from the cytoplasmic fraction of the strictly anaerobic, gram-negative *Dsp. multivorans* (Neumann et al. 1996). The protein catalyzes the reductive dehalogenation of tetrachloroethene and trichloroethene to *cis*-1,2-dichloroethene with methyl viologen as the artificial electron donor and contains a corrinoid as well as iron and acid-labile sulfur as prosthetic groups. The dehalogenation activity is stimulated by the addition of ammonium ions and is severely inhibited by the addition of chlorinated methanes (Neumann et al. 1996). In addition, a membrane-bound PCE dehalogenase of *"Dba. restrictus"* has been isolated (Schumacher et al. 1997). The enzyme seems to be very similar to the enzyme of *Dsp. multivorans* with respect to the molecular mass and cofactor content. Here we report on the purification and characterization of a membrane-associated PCE reductive dehalogenase from the strictly anaerobic, gram-positive strain PCE-S.

Materials and methods

Purification of the tetrachloroethene dehalogenase

Strain PCE-S was grown anaerobically on a medium containing pyruvate and fumarate as described elsewhere (Miller et al. 1997 b). The bacteria were harvested in the late exponential growth phase by centrifugation at 5,000 × *g* and at 4 °C for 20 min, were frozen in liquid nitrogen, and were stored frozen at –20° C under aerobic conditions. Cell pellets were resuspended in 10 mM Tris-HCl (pH 7.5; 4 ml per 1 g pellet) containing 0.5 mM dithiothreitol, 0.1% (v/v) Triton X-100 (reduced form), 2 mM 4-(2-aminoethyl)-benzene-sulfofluoride, 10 mg lysozyme ml^{-1}, and 0.3 mg DNase I ml^{-1} and were incubated for 45 min at 37 °C. After addition of EDTA and Triton X-100 (reduced form) to final concentrations of 1 mM and 0.15% (v/v), respectively, the cell extract was incubated at 4 °C for 2 h. Cell debris was removed by centrifugation under a N_2/H_2 (95%/5%; v/v) atmosphere for 1 h at 35,000 × *g* and 4 °C. The resulting supernatant was applied to a Q-Sepharose HP column (1.6 × 10 cm) pre-equilibrated with Mops buffer [20 mM Mops-KOH (pH 6.5) containing 0.5 mM dithiothreitol and 0.1% Triton X-100 (reduced form)]. The dehalogenase was eluted with a linear gradient from 0 to 0.4 M KCl in Mops buffer at a KCl concentration of approximately 0.13 M. The fraction containing the highest dehalogenase activity was passed through a Superdex 200 column (1.6 × 60 cm) pre-equilibrated with Mops buffer. Dehalogenase-containing fractions were pooled and applied to a Mono Q column (1.0 × 10 cm) pre-equilibrated with Mops buffer. The enzyme was eluted with a linear gradient from 0 to 0.5 M KCl in Mops buffer at a KCl concentration of approximately 0.1 M. All steps were performed in an anaerobic chamber with N_2/H_2 (95%/5%, v/v) as the gas phase.

Analytical methods

PCE dehalogenase and trichloroethene (TCE) dehalogenase activities were routinely assayed by photometric determination of the oxidation of reduced methyl viologen with tetrachloroethene or trichloroethene as electron acceptors at 578 nm (ε_{578} = 9.7 mM^{-1} cm^{-1}) and 25° C. The assay was conducted in 100 mM Tris-HCl (pH 7.5) containing 0.5 mM methyl viologen and 0.5 mM of the chlorinated hydrocarbon in rubber-stoppered glass cuvettes with N_2 (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 titanium(III) citrate solution (Zehnder and Wuhrmann 1976). The reaction was started by the addition of protein. The velocity was determined by calculating 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 as described elsewhere (Neumann et al. 1996). The assays were conducted at 700 nm (ε_{700} = 1.6 $mM^{-1}cm^{-1}$) for methyl viologen concentrations up to 1.5 mM, and at 760 nm (ε_{760} = 1.25 mM^{-1} cm^{-1}) for higher methyl viologen concentrations. The data for the enzyme kinetics were fitted to Michaelis-Menten kinetics (assuming substrate inhibition for trichloroethene), and the kinetic parameters (K_m and K_i) were calculated using a computer program (GraphPad Prism; GraphPad Software, San Diego, Calif., USA).

The pH dependence of the tetrachloroethene dehalogenase activity was tested by varying the buffer components in the assay. Sodium acetate was used in the range from pH 4.4 to pH 5.4, Tris-maleate from pH 5.2 to pH 7.2, and Tris-HCl from pH 7.0 to pH 9.0. The temperature dependence of the enzyme was determined by incubating the PCE dehalogenase (0.25 mg protein per ml Mops buffer) for 5 min at a temperature ranging from 20 to 95 °C and subsequent determination of the dehalogenase activity using the photometric assay described above at the same temperature. Oxygen sensitivity was determined by stirring PCE dehalogenase (0.25 mg protein per ml basic buffer) in the presence of air at 4 °C.

The molecular mass of the native protein was determined in the presence of Triton X-100 (reduced form) and 0.15 M KCl by gel-filtration chromatography on Superdex 200 as described in the purification procedure. The standards (in kDa) were thyroglobulin (669), ferritin (440), catalase (232), aldolase (158), bovine serum albumin (67), chymotrypsinogen (25), and myoglobin (17.8). The standard proteins were also run in the presence of Triton X-100. The molecular mass of the subunits and the subunit composition were determined using an SDS-polyacrylamide gel (12%) that was subsequently silver-stained. The standard proteins (in kDa) were phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20.1), and α-lactalbumin (14.4).

The protein concentration was determined according to Bradford (1976) with the BioRad Protein Assay using bovine serum albumin as the standard. Iron was assayed as described by Fish (1988) using an iron volumetric standard. Acid-labile sulfide was determined according to Rabinowitz (1978). The corrinoid content of the dehalogenase was calculated by spectrophotometrical quantitation ($\Delta\varepsilon_{580-640}$ = 7.3 mM^{-1} cm^{-1}) of the cyanide-extracted corrinoids (2 mg KCN/ml) using hydroxocobalamin as the standard (Fischer et al. 1992). The determination of the cobalt and iron content was kindly performed by R. Hedderich and J. Koch (Max-Planck-Institut für terrestrische Mikrobiologie, Marburg, Germany) using atomic absorption spectroscopy.

Source of materials

Tetrachloroethene was purchased from Merck (Darmstadt, Germany). Trichloroethene was obtained from Ferak (Berlin, Germany). An iron volumetric standard was purchased from Aldrich (Steinheim, Germany), and Triton X-100 (reduced form) was from Fluka (Neu-Ulm, Germany). Gases [CO (grade 4.7), CO_2 (grade 4.5), N_2/H_2 (95%/5%, v/v), and N_2 (grade 4.6)] were supplied by Messer Griesheim (Düsseldorf, Germany). All chemicals used were of the highest available purity and were purchased from Aldrich (Steinheim, Germany), Boehringer (Mannheim, Ger-

many), Fluka (Neu-Ulm, Germany), Merck, and Sigma (Deisenhofen, Germany). BioRad Protein Assay was from Bio-Rad Laboratories (Munich, Germany).

Results

Purification of the tetrachloroethene dehalogenase

The tetrachloroethene reductive dehalogenase was purified from pyruvate- and fumarate-grown cells of strain PCE-S. Since the dehalogenase is membrane-associated (Miller et al. 1997b), the detergent Triton X-100 was added to extract the enzyme completely from the membrane fraction. In addition, Triton X-100 was added throughout the purification procedure to improve the resolution of the chromatographic steps and to increase the yield of dehalogenase during the isolation of the enzyme. The purification procedure is summarized in Table 1. The PCE dehalogenase was judged to be homogenous on the basis of SDS-PAGE (Fig. 1). The specific activity of the purified enzyme was determined with methyl viologen (0.5 mM) as electron donor to be approximately 650 nkat/mg protein with a yield of nearly 75% throughout the purification procedure. The enzyme was oxygen-sensitive and lost approximately 50% of its activity during incubation and stirring in the presence of air for 50 min at 4 °C. ThepH optimum of the tetrachloroethene dehalogenation activity was found to be approximately 7.2 (data not shown). The highest enzymatic activity was obtained at a temperature of 50° C (data not shown).

Gel filtration in the presence of Triton X-100 on Superdex 200 indicated an apparent molecular mass for the native PCE dehalogenase of approximately 200 kDa (Fig. 2). SDS-PAGE revealed a single protein band, indicating that the dehalogenase consists of one subunit. The apparent molecular mass of the subunit was calculated to be 65 kDa (Fig. 1).

The iron content of the dehalogenase was determined to be 7.8 ± 0.5 mol iron/mol subunit. The content of acid-labile sulfur was estimated to be 10.3 ± 2 mol sulfur/mol subunit. The cobalt content of the enzyme was determined by atomic absorption spectroscopy to be 1.0 ± 0.3 mol cobalt/mol subunit. Extraction of PCE dehalogenase by incubation of the enzyme for 10 min at 100 °C in the presence of cyanide revealed an absorption spectrum of the

Table 1 Purification of tetrachloroethene dehalogenase from strain PCE-S. In this preparation, 5 g cells (wet wt.) was used. For experimental detail, see Materials and methods. (*1 nkat* 1 nmol chloride released or 2 nmol methyl viologen oxidized per s at 25 °C)

Purification step	Total activity (nkat)	Yield (%)	Specific activity (nkat/mg protein)	Purification factor
Crude extract	1,100	100	4	1
Q-Sepharose	1,100	100	190	50
Superdex 200	1,100	100	425	110
Mono Q	800	75	650	165

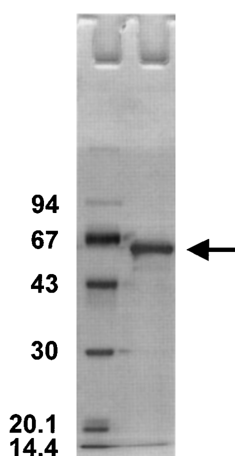

Fig. 1 SDS-PAGE of purified tetrachloroethene reductive dehalogenase from strain PCE-S. Enzyme (2.5 µg) was electrophoresed in the presence of 0.1% SDS (*lane 2*). The gel was silver-stained. Molecular mass markers (*lane 1*; mass shown in kDa) were phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20.1), and α-lactalbumin (14.4). The *arrow* indicates the dehalogenase band

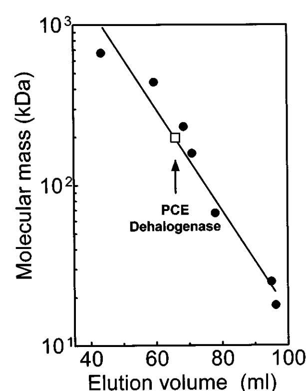

Fig. 2 Determination of the apparent molecular mass of the native tetrachloroethene reductive dehalogenase by gel filtration on Superdex 200 (1.6 × 60 cm). The column was eluted with 20 mM Mops-KOH (pH 6.5) containing 0.15 M KCl, 0.5 mM dithiothreitol, and 0.1% Triton X-100 at a rate of 1 ml/min. Standards (mass shown in kDa): thyroglobulin (669), ferritin (440), catalase (232), aldolase (158), bovine serum albumin (67), chymotrypsinogen (25), and myoglobin (17.8)

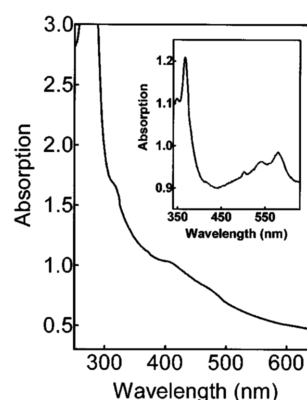

Fig. 3 UV/visible absorption spectrum of the purified tetrachloroethene reductive dehalogenase (1 mg/ml) in 20 mM Mops-KOH (pH 6.5) containing 0.1 M KCl, 0.5 mM dithiothreitol, and 0.1% Triton X-100, and the UV/visible absorption spectrum of dicyano-cob(III)alamin extracted from the enzyme (*inset*). For experimental details see Materials and methods

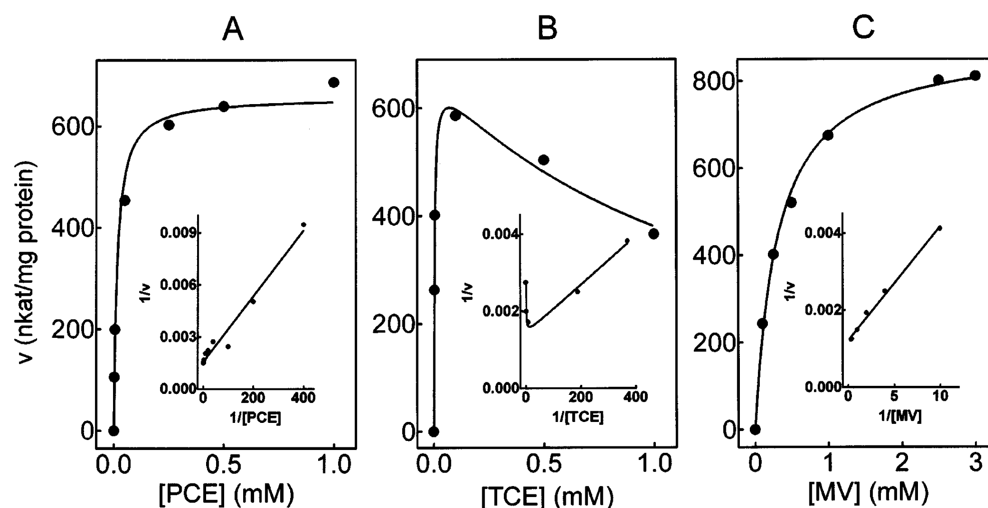

Fig. 4 Tetrachloroethene reductive dehalogenase activity as a function of the **A** tetrachloroethene (*PCE*), **B** trichloroethene (*TCE*), and **C** methyl viologen (*MV*) concentration; the inset shows the Lineweaver-Burk plots. The reactions were started by the addition of approximately 3.0 μg PCE dehalogenase. For details on the experimental conditions, see Materials and methods

extract typical for dicyano-cob(III)alamin (inset in Fig. 3). From the absorption difference A_{580}–A_{650}, the corrinoid content of the enzyme was estimated to be 0.7 mol corrinoid/mol subunit using dicyano-cobalamin as the standard (Fischer et al. 1992).

The UV/visible spectrum of the PCE dehalogenase exhibited absorption maxima near 280 and shoulders at 400 nm and at 310 nm (Fig. 3). The absorption maximum at 400 nm was probably due to the presence of iron-sulfur clusters. Typical absorption maxima for cob(I)alamin (385 nm) or cob(III)alamin (360 nm) could not be detected in the UV/visible spectrum (Fig. 3). The shoulder in the absorption spectrum at approximately 310 nm may be taken to indicate the presence of a corrinoid in the cob(II)alamin form (Fig. 3).

The apparent K_m values for PCE and TCE were determined to be 10 μM and 4 μM, respectively, at a methyl viologen concentration of 1.6 mM (Fig. 4 A, B). The corresponding V_{max} values at this nonsaturating methyl viologen concentration were approximately 650 and 690 nkat/mg protein, respectively. Higher concentrations of TCE inhibited the PCE dehalogenase ($K_i \approx 1.3$ mM). The apparent K_m for methyl viologen was determined to be 0.3 mM at a PCE concentration of 1 mM (Fig. 4 C); the V_{max} value was near 850 nkat/mg protein.

Besides tetrachloroethene, only trichloroethene was reductively dechlorinated by the enzyme; the reaction rate was approximately 70% of the tetrachloroethene dehalogenation rate. Carbon tetrachloride, 1,1-dichloroethene, *trans*-1,2-dichloroethene, *cis*-1,2-dichloroethene, 3-chlorobenzoate, 3-chloro-4-hydroxyphenol, pentachlorophenol, 2,4,6-trichlorophenol and 2,4,5-trichlorophenol were not converted by the enzyme at significant reaction rates (data not shown). The addition of ammonium ions did not affect the reaction velocity of tetrachloroethene dehalogenase (data not shown).

N-terminal amino acid sequencing of the purified PCE dehalogenase using Edman degradation revealed the following sequence: ADIVAPITETSEFPYKVDAY. No significant sequence similarities to the deduced amino acid sequence from the PCE reductive dehalogenase gene of *D. multivorans* (A. Neumann, G. Wohlfarth and G. Diekert; unpublished work) were detected using the alignment utility of the software package PC/Gene (IntelliGenetics, Geneva, Switzerland).

Discussion

The tetrachloroethene dehalogenase of the gram-positive strain PCE-S mediates in vitro the reductive dehalogenation of tetrachloroethene to trichloroethene and further to *cis*-1,2-dichloroethene with methyl viologen as an artificial electron donor at catabolic rates. The finding that PCE dehalogenase activity is similar in crude extracts of pyruvate/fumarate- and in pyruvate/PCE-grown cells indicates that the enzyme is constitutively expressed in the bacterium. The dehalogenase appears to be membrane-associated (Miller et al. 1997 b) and had to be purified in the presence of the detergent Triton X-100 to obtain a higher yield of enzymatic activity. In contrast, PCE reductive dehalogenase of the gram-negative bacterium *Dsp. multivorans* could be purified in the absence of a detergent from the cytoplasmic fraction (Neumann et al. 1996).

The K_m values of the purified strain PCE-S enzyme for PCE and TCE were approximately one-tenth of the respective values of the enzyme of *Dsp. multivorans*. Since lower threshold values are achievable, strain PCE-S is probably more suitable for PCE remediation than is *Dsp. multivorans*, although additional factors may be important for the application of a bacterium for bioremediation. Both enzymes exhibited substrate inhibition by TCE, although the K_i for the enzyme of *Dsp. multivorans* was approximately tenfold higher. At present, the physiological electron donor of the enzyme is not known. Neither other chloroethenes nor chloromethanes and chlorinated aromatic compounds are able to serve as substrates for the enzyme, indicating a high substrate specificity of the tetrachloroethene dehalogenase of strain PCE-S and suggesting that different enzymes may be responsible for the re-

Table 2 Comparison of the properties of tetrachloroethene (PCE) dehalogenases from strain PCE-S and *Dehalospirillum multivorans* (Neumann et al. 1996) (*MV* methyl viologen)

Properties of PCE dehalogenase	Strain PCE-S	*Dehalospirillum multivorans*
Apparent molecular mass (kDa)		
SDS-PAGE	65	57
Gel filtration	200	58
Cobalt (mol/mol subunit)	1.0 ± 0.3	1.0 ± 0.3
Corrinoid (mol/mol subunit)	0.7 ± 0.3	1.1 ± 0.3
Fe (mol/mol subunit)	7.8 ± 0.5	9.8 ± 2.0
Acid-labile S (mol/mol subunit)	10.3 ± 2.0	8.0 ± 1.5
V_{max} (25 °C, 0.5 mM MV; kat/mg protein)	650	2,650
Apparent K_m (µM)		
Tetrachloroethene	10	200
Trichloroethene	4	240
Methyl viologen	300	300
Oxygen stability ($t_{1/2}$ in min)	50	120
Temperature optimum (°C)	50	42
pH optimum	7.2	8.0
Stimulation by NH_4^+	–	+

ductive dechlorination of chlorinated aliphatic and aromatic compounds in *Desulfitobacterium* strains. PCE dehalogenase of *Dsp. multivorans* exhibits a similar substrate specificity (Neumann et al. 1996), which is not surprising since *Dsp. multivorans* is not able to convert chlorinated aromatic compounds. In contrast, the PCE dechlorination activity of *Desulfomonile tiedjei* is probably a secondary activity of the 3-chlorobenzoate reductive dehalogenase, as is indicated by similar activities with both substrates and similar inhibition and inactivation patterns in cell extracts (Townsend and Suflita 1996).

The tetrachloroethene dehalogenase consists of a single subunit with an apparent molecular mass of 65 kDa, which is similar to that of the monomeric 57-kDa enzyme of *Dsp. multivorans* (Table 2). In contrast to *Dsp. multivorans*, the native dehalogenase of strain PCE-S appears to exhibit a trimeric structure as indicated by the data obtained by gel filtration (Table 2). Since it is known that membrane proteins tend to form aggregates due to their high hydrophobicity, the apparent molecular mass of 200 kDa for the native PCE dehalogenase of strain PCE-S may be a purification artifact, and the enzyme could be essentially monomeric in vivo. PCE dehalogenase contained 1 mol corrinoid per mol subunit as indicated by cobalt determination and extraction of dicyano-cobalamin (due to the extraction conditions) from the enzyme. In addition, the detection of approximately 8 mol Fe and 10 mol S (Table 2) may be taken to indicate the presence of 2 Fe4–S4 clusters as additional cofactors. This was further supported by the detection of the same prosthetic groups in the enzyme of *Dsp. multivorans* (Neumann et al. 1996; Table 2) and the identification of a binding motif for 2 Fe4–S4 clusters in the gene of the PCE dehalogenase of this organism (A. Neumann, G. Wohlfarth and G. Diekert, unpublished work). Recently, a PCE reductase of the strictly anaerobic, gram-positive *"Dba. restrictus"* was purified from the membrane fraction of the cell extract. The specific activity of the enzyme was approximately 200 nkat/mg, i.e. approximately one third of the PCE-S enzyme and approximately one tenth of the *Dps. multivorans* enzyme. This 60-kDa protein contained 1 corrinoid, 7.1 iron, and 9.3 sulfur as prosthetic groups and exhibited a similar UV-visible spectrum (Schumacher et al. 1997; W. Schumacher and C. Holliger, EAWAG, Kastanienbaum, Switzerland, personal communication). Evidence for the presence of two Fe4-S4 clusters was provided by electron spin resonance measurements (Schumacher et al. 1997). The cofactor composition of PCE dehalogenase of strain PCE-S and the comparison with the PCE-dechlorinating enzymes *of Dsp. multivorans* and *"Dba. restrictus"* further support the assumption that the enzyme is probably a monomeric 65-kDa protein.

The first amino acid of the N-terminus of PCE dehalogenase was alanine instead of methionine, indicating a post-translational processing of the enzyme. This is in accordance with the membrane association of the dehalogenase since membrane proteins are often synthesized as preproteins with a leader peptide at the N-terminus that is removed during maturation of the protein. The absence of any significant sequence similarity of the N-terminal peptide to the PCE dehalogenase of *Dsp. multivorans* may be taken to indicate an independent evolution of the tetrachloroethene dehalogenases in the gram-negative *Dsp. multivorans* and the gram-positive strain PCE-S. To support this assumption and to investigate the evolutionary origin of the dehalogenating enzymes, cloning and sequencing of the gene from strain PCE-S are required. These procedures are currently under progress in our laboratory.

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