Purification and characterization of the 3-chloro-4-hydroxy-phenylacetate reductive dehalogenase of *Desulfitobacterium hafniense*

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Abstract The membrane-bound 3-chloro-4-hydroxyphenylacetate (Cl-OHPA) reductive dehalogenase from the chlorophenol-reducing anaerobe *Desulfitobacterium hafniense* was purified 11.3-fold to apparent homogeneity in the presence of the detergent CHAPS. The purified dehalogenase catalyzed the reductive dechlorination of Cl-OHPA to 4-hydroxyphenylacetate with reduced methyl viologen as the electron donor at a specific activity of 103.2 nkat/mg protein. SDS-PAGE revealed a single protein band with an apparent molecular mass of 46.5 kDa. The enzyme contained 0.68 ± 0.2 mol corrinoid, 12.0 ± 0.7 mol iron, and 13.0 ± 0.7 mol acid-labile sulfur per mol subunit. The N-terminal amino acid sequence of the enzyme was determined and no significant similarity was found to any protein present in the gene bank.

Key words: 3-Chloro-4-hydroxyphenylacetate reductive dehalogenase; Corrinoid protein; Iron-sulfur protein; N-terminal amino acid sequence; Tetrachloroethene dehalogenase; *Desulfitobacterium hafniense*

1. Introduction

Desulfitobacterium is a genus of strictly anaerobic, endospore-forming, Gram-positive bacteria. The organisms belonging to this genus are capable of reductive dechlorination of various chlorinated hydrocarbons including chlorinated phenols and/or alkenes in their energy metabolism [1,2]. Alternatively, most of the *Desulfitobacterium* species can utilize sulfite and thiosulfate but not sulfate as terminal electron acceptor.

Desulfitobacterium hafniense formerly named strain DCB-2 was enriched from a trichlorophenol-converting consortium, which was isolated from municipal sludge [3]. The bacterium is able to reductively dechlorinate 3-chloro-4-hydroxyphenylacetate to 4-hydroxyphenylacetate and 2,4,6-trichlorophenol via 2,4-dichlorophenol to 4-chlorophenol with pyruvate as the electron donor.

The first reductive dehalogenase reported to be purified was the membrane-bound 3-chlorobenzoate dechlorinating enzyme from the Gram-negative, sulfate-reducing *Desulfomonile tiedjei* [4]. In addition, reductive dehalogenases dechlorinating tetrachloroethene (PCE) via trichloroethene to *cis*-1,2-dichloroethene were purified from the Gram-negative *Dehalospirillum multivorans* [5] and the Gram-positives *Dehalobacter restrictus* [6] and *Desulfitobacterium* strain PCE-S [7]. While UV-visible spectra of the 3-chlorobenzoate reductive dehalogenase suggested a heme cofactor [4], the PCE dehalogenating enzymes purified so far were found to contain a corrinoid and iron-sulfur clusters as prosthetic groups [5–7]. Here we report on the purification and characterization of a corrinoid- and Fe/S-containing membrane-bound 3-chloro-4-hydroxyphenylacetate reductive dehalogenase from the strictly anaerobic bacterium *Desulfitobacterium hafniense*.

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Abbreviations: Cl-OHPA, 3-chloro-4-hydroxyphenylacetate; PCE, tetrachloroethene

2. Materials and methods

2.1. Purification of the 3-chloro-4-hydroxyphenylacetate reductive dehalogenase

Desulfitobacterium hafniense was isolated from municipal sludge [3,8]. The organism was grown anaerobically according to [9]. The medium contained 15 mM 3-chloro-4-hydroxyphenylacetate and 15 mM pyruvate as energy sources. The bacteria were harvested in late exponential growth phase by centrifugation. Cells (1 g wet weight) were resuspended in 3 ml standard buffer (50 mM Tris-HCl, pH 8.0, containing 1 mM dithiothreitol (DTT)) and disrupted by passing the suspension twice through a French Press cell at 137 MPa under anaerobic conditions. The suspension was centrifuged for 15 min at 10 000 × *g* and 4°C. The pellet was resuspended in 3 ml standard buffer and disrupted again by passing the suspension twice through a French Press cell under anaerobic conditions. After centrifugation of the suspension, the supernatants were pooled and protamine sulfate was added to a final concentration of 0.5 mg/ml. The precipitate was removed by centrifugation for 15 min at 10 000 × *g* and 4°C. The membrane fraction was obtained by centrifugation for 120 min at 150 000 × *g* and 4°C and resuspended in standard buffer containing 17.4% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 50 mM CHAPS. The dehalogenase was extracted by stirring the suspension under anaerobic conditions at 0°C and centrifugation for 20 min at 150 000 × *g* and 4°C. The supernatant was applied to a Mono Q column (1 × 10 cm) pre-equilibrated with buffer A (50 mM Tris-HCl, pH 8, 17.4% glycerol, 1 mM DTT, 6 mM CHAPS). The enzyme was eluted with a linear gradient from 0 to 1.5 M KCl in buffer A at a KCl concentration between 0.30 M and 0.48 M. The fraction containing the highest dehalogenase activity was supplemented with $(NH_4)_2SO_4$ to a final concentration of 1.0 M and passed through a phenyl-Superose column (0.5 × 5 cm) pre-equilibrated with buffer A containing 1.0 M $(NH_4)_2SO_4$. The enzyme was eluted with a linear gradient from 1.0 to 0 M $(NH_4)_2SO_4$ in buffer A at a $(NH_4)_2SO_4$ concentration between 0.15 M and 0 M. All steps were performed in an anaerobic chamber with N_2/H_2 (95:5%; v/v) as the gas phase.

2.2. Analytical methods

The dehalogenase activity was routinely assayed by photometric determination of the oxidation of reduced methyl viologen in Cl-OHPA as electron acceptor at 578 nm ($\varepsilon_{578} = 9.7\ mM^{-1}\ cm^{-1}$). The assay was conducted with 100 mM Tris-HCl, pH 7.5, containing 0.5 mM methyl viologen in rubber-stoppered glass cuvettes with N_2 as the gas phase. Methyl viologen was reduced by the addition of titanium(III) citrate solution [10]. After addition of protein, the reaction was started with 1 mM Cl-OHPA. The reaction velocity was calculated from the rate of the decrease of the absorption between 2.5 and 2.0.

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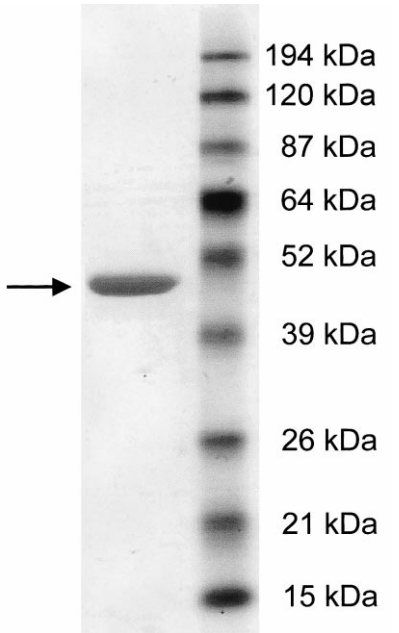

Fig. 1. SDS-PAGE of the purified 3-chloro-4-hydroxyphenylacetate reductive dehalogenase from *Desulfitobacterium hafniense*. The enzyme (9 μg) was electrophoresed in the presence of 12% SDS (lane 2). The gel was stained with Coomassie Blue G-250. Molecular mass markers (lane 1; in kDa) were phosphorylase *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.

The apparent molecular mass of the dehalogenase was determined using SDS-PAGE (12%); the gel was stained with Coomassie Blue G-250. The standard proteins were Benchmark prestained protein ladder (Life Technologies, Karlsruhe, Germany).

Isoelectric focusing was done using an Ampholine-PAGE with 1 M NaOH as cathode solution and 1 M H_3PO_4 as anode solution. The gel was run on a Multiphor II, Pharmacia. The standard proteins were broad range IEF standards from Biorad (p*I* 4.45–9.6).

The protein concentration was determined according to [11] with the Bio-Rad Protein Assay using bovine serum albumin as the standard. Iron was assayed as described by Fish [12] using an iron volumetric standard. Acid-labile sulfide was determined according to [13]. The corrinoid content of the dehalogenase was calculated from the absorption spectrum of the cyanide-extracted corrinoids [14].

2.3. Source of materials

The iron volumetric standard was purchased from Aldrich (Steinheim, Germany) and CHAPS from Gerbu Biotechnik (Gaiberg, Germany). Gases (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, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), and Sigma (Deisenhofen, Germany). Bio-Rad Protein Assay was from Bio-Rad (München, Germany).

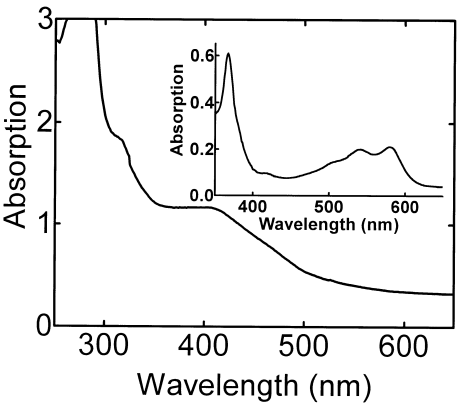

Fig. 2. UV/visible absorption spectrum of the purified 3-chloro-4-hydroxyphenylacetate reductive dehalogenase (1 mg/ml) in buffer A and the UV/visible absorption spectrum of dicyano-cob(III)alamin extracted from the enzyme (inset). For experimental details see Section 2.

3. Results

Desulfitobacterium hafniense induced a 3-chloro-4-hydroxyphenylacetate (Cl-OHPA) reductive dehalogenase upon growth on Cl-OHPA-containing media. When the cells were grown in the absence of Cl-OHPA, e.g. in a pyruvate- and fumarate-containing medium, no dehalogenation activity could be detected. The Cl-OHPA reductive dehalogenase was purified from pyruvate- and Cl-OHPA-grown cells of *D. hafniense*. The enzyme was found exclusively in the particulate fraction of the cells and was extracted from the membrane using the detergent CHAPS (50 mM). 0.5% Triton X-100 (reduced) could also be used to extract the dehalogenase. To avoid precipitation of the ammonium sulfate, CHAPS was added throughout the purification of the dehalogenase. The purification procedure is summarized in Table 1. The Cl-OHPA reductive dehalogenase was judged to be homogeneous on the basis of SDS-PAGE (Fig. 1). 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 46.5 kDa (Fig. 1). The isoelectrical point was determined to be lower than 4.45.

The dehalogenating enzyme catalyzed the reductive dechlorination of Cl-OHPA with reduced methyl viologen as artificial electron donor. Tetrachloroethene was not dehalogenated at significant rates. The specific activity of the purified enzyme was determined with methyl viologen (0.5 mM) as electron donor to be approximately 103.2 nkat/mg protein with a yield of 18% throughout the purification procedure. Addition of ammonium sulfate (4.3 mM) to the assay resulted in a 40% stimulation of the dechlorination activity.

Table 1
Purification of the 3-chloro-4-hydroxyphenylacetate reductive dehalogenase of *Desulfitobacterium hafniense* (5.5 g cells wet weight)

Purification step	Total activity (nkat[a])	Yield (%)	Specific activity (nkat/mg protein)	Purification factor
Crude extract	771	100	9.1	1
CHAPS extraction	462	60	3.5	0.4
Mono Q	254	33	32.0	3.5
Phenyl Superose	148	18	103.2	11.3

For experimental detail, see Section 2.
[a]1 nkat is defined as 1 nmol chloride released or 2 nmol methyl viologen oxidized per second.

Table 2
Properties of reductive dehalogenases

	Enzyme				
	Cl-OHPA reductive dehalogenase	3-Cl-benzoate reductive dehalogenase (4)	PCE reductive dehalogenase (7)	PCE reductive dehalogenase (6)	PCE reductive dehalogenase (7)
Organism	*Desulfitobacterium hafniense*	*Desulfomonile tiedjei*	*Desulfitobacterium* strain PCE-S	*Dehalobacter restrictus*	*Dehalospirillum multivorans*
Substrate	Cl-OHPA	3-Cl-benzoate	PCE, TCE	PCE, TCE	PCE, TCE
Localization	Membrane	Membrane	Membrane	Membrane	Cytoplasm[a]
SDS-PAGE (kDa)	47	64 (α); 37 (β)	65	60	57
Apparent K_m (μM)	xx	n.d.	10 (PCE)	20 (PCE)	200 (PCE)
Specific activity (nkat/mg)	103	300	650	234	2640
Cofactors (mol/mol subunit)	1 corrinoid, 12 Fe, 13 S	Heme, Fe/S: n.d.	1 corrinoid, 8 Fe, 8 S	1 corrinoid, 8 Fe, 8 S	1 corrinoid, 8 Fe, 8 S

[a]Catalytically active subunit; Cl-OHPA = 3-chloro-4-hydroxyphenylacetate; n.d. = not determined; PCE = tetrachloroethene; TCE = trichloroethene.

The iron content of the dehalogenase was determined to be 12.0 ± 0.7 mol iron/mol subunit. The content of acid-labile sulfur was estimated to be 13.0 ± 0.7 mol sulfur/mol subunit. The UV/visible spectrum of the PCE dehalogenase exhibited absorption maxima near 280 and 400 nm and shoulders at 310 nm (Fig. 2). 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. The shoulder in the absorption spectrum at about 310 nm may be taken to indicate the presence of a corrinoid in the cob(II)alamin form. Cyanide extraction of Cl-OHPA reductive dehalogenase revealed an absorption spectrum of the extract typical for dicyano-cob(III)alamin (inset in Fig. 2). From the absorption difference A_{580}–A_{670}, the corrinoid content of the enzyme was estimated to be 0.7 ± 0.2 mol corrinoid/mol subunit using dicyano-cobalamin as the standard [14]. Surprisingly, propyl iodide, which is known to light-reversible inactivate corrinoid-containing enzymes [5], did not inhibit the Cl-OHPA reductive dehalogenase.

N-terminal amino acid sequencing of the purified Cl-OHPA reductive dehalogenase using Edman degradation revealed the sequence AETLNYVPGSGKIRSKLRPVHDFA. The deduced amino acid sequence from the tetrachloroethene reductive dehalogenase gene of *Dehalospirillum multivorans* and to the N-terminus of the *Desulfitobacterium* strain PCE-S enzyme showed no significant sequence similarities to this sequece when using the alignment utility of the software package PC/Gene (IntelliGenetics, Geneva, Switzerland).

4. Discussion

The 3-chloro-4-hydroxyphenylacetate (Cl-OHPA) reductive dehalogenase of the Gram-positive *Desulfitobacterium hafniense* mediates in vitro the reductive dechlorination of Cl-OHPA to 4-hydroxyphenylacetate with reduced methyl viologen as the artificial electron donor. At present, the physiological electron donor of the enzyme is not known. The enzyme is membrane-bound as is the 3-chlorobenzoate (3CB) reductive dehalogenase of the Gram-negative *Desulfomonile tiedjei*, which catalyzes a similar reaction, namely the reductive dechlorination of 3-chlorobenzoate to benzoate [4]. Whereas 3CB reductive dehalogenase also dechlorinates tetrachloroethene (PCE) via trichloroethene (TCE) to *cis*-1,2-dichloroethene (DCE) in a 'side reaction' [15], Cl-OHPA dehalogenase does not convert PCE at significant rates. In comparison to the *Desulfomonile* enzyme, which consists of two subunits with apparent molecular masses of 64 and 37 kDa [4], SDS-PAGE revealed the presence of a single subunit with an apparent molecular mass of 47 kDa for the Cl-OHPA dehalogenase. In addition, both enzymes contained different cofactors. While the Cl-OHPA dehalogenase contains a corrinoid and iron-sulfur centers, the 3-chlorobenzoate dehalogenase (4) probably binds a heme as prosthetic group (Table 2).

With regard to the cofactors involved in reductive dechlorination, Cl-OHPA dehalogenase of *D. hafniense* is similar to the tetrachloroethene reductive dehalogenases described so far, namely the enzymes of *Dehalospirillum multivorans* [5], *Dehalobacter restrictus* [6], and *Desulfitobacterium* strain PCE-S [7] (Table 2). These enzymes contain one corrinoid and iron-sulfur centers as cofactors and reductively dechlorinate PCE via TCE to DCE with reduced methyl viologen as the artificial electron donor. Aryl halides are not converted by the PCE dehalogenases [7]. From the finding that approximately 12 mol Fe and 12 mol acid-labile sulfur were detected per mol subunit, it may be concluded that the enzyme might contain 3 Fe_4S_4 centers, while 2 tetranuclear Fe/S centers were described for the PCE dehalogenating enzymes. The stimulatory effect of ammonium sulfate on the Cl-OHPA-dechlorination cannot be explained at present. It should be noted, however, that the PCE reductive dehalogenase of *D. multivorans* was also reported to be stimulated by NH_4^+ [5], whereas the enzyme of strain PCE-S was not affected [7] and the *D. restrictus* enzyme was slightly inhibited by this ion [6].

The first amino acid of the N-terminus of Cl-OHPA reductive dehalogenase was alanine instead of methionine, indicating a post-translational processing of the enzyme. This is in accordance with the localization of the enzyme in the particulate fraction, since membrane proteins are often synthesized as preproteins with a leader peptide at the N-terminus, which is removed during maturation of the protein. The absence of any significant sequence similarity of the N-terminal peptide to the PCE dehalogenases of *D. multivorans* [16], *D. restrictus* (C. Holliger, personal communication), and strain PCE-S [7] may be taken to indicate an evolution of the Cl-OHPA reduc-

tive dehalogenase independent from that of the tetrachloroethene dehalogenases.

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