JOURNAL OF BACTERIOLOGY, May 1985, p. 676–681
0021-9193/85/050676-06$02.00/0
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Vol. 162, No. 2

Dichloromethane Dehalogenase of *Hyphomicrobium* sp. Strain DM2

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Received 6 November 1984/Accepted 28 January 1985

Dichloromethane dehalogenase, a highly inducible glutathione-dependent enzyme catalyzing the conversion of dichloromethane into formaldehyde and inorganic chloride, was purified fivefold with 60% yield from *Hyphomicrobium* sp. strain DM2. The electrophoretically homogeneous purified enzyme exhibited a specific activity of 17.3 mkat/kg of protein. Its pH optimum was 8.5. The enzyme was stable at −20°C for at least 6 months. A subunit molecular weight of 33,000 was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel filtration of native dichloromethane dehalogenase yielded a molecular weight of 195,000. Subunit cross-linking with dimethyl suberimidate confirmed the hexameric tertiary structure of the enzyme. Dichloromethane dehalogenase was highly specific for dihalomethanes. Its apparent K_m values were 30 μM for CH_2Cl_2, 15 μM for CH_2BrCl, 13 μM for CH_2Br_2, 5 μM for CH_2I_2, and 320 μM for glutathione. Several chlorinated aliphatic compounds inhibited the dichloromethane dehalogenase activity of the pure enzyme. The K_i values of the competitive inhibitors 1,2-dichloroethane and 1-chloropropane were 3 and 56 μM, respectively.

Bacteria using dichloromethane as the carbon and energy source have been shown to occur in sewage sludge (10) or in soil exposed to this xenobiotic for extended periods of time (17, 21). Pure cultures of dichloromethane-degrading bacteria were isolated from such habitats and identified as facultative methylotrophs of the genera *Pseudomonas* (3, 12) and *Hyphomicrobium* (22). To grow on dichloromethane these organisms thus must be able to convert dichloromethane into formaldehyde and inorganic chloride. An inducible enzyme catalyzing this conversion in a glutathione-dependent reaction indeed was demonstrated in crude extracts of *Hyphomicrobium* sp. strain DM2 (22). By analogy with the glutathione-dependent metabolism of dichloromethane in rat liver cytosol (1), the formation of formaldehyde was thought to proceed through the following intermediates.

$$CH_2Cl_2 \xrightarrow[HCl]{GSH} [GS\text{-}CH_2Cl] \xrightarrow[HCl]{Water} GS\text{-}CH_2OH \rightleftarrows CH_2O + GSH$$

GSH and GS represent free and bound glutathione, respectively. In this sequence of reactions an *S*-chloromethyl glutathione conjugate is formed enzymatically which is assumed to undergo nonenzymatic hydrolysis to yield *S*-hydroxymethyl glutathione. Decomposition of the latter compound then leads to formaldehyde and to the regeneration of reduced glutathione. Evidence that dichloromethane dehalogenation in *Hyphomicrobium* sp. strain DM2 proceeds by this mechanism is based on the stoichiometry of the reaction in crude extracts (22) and on the fact that the protons of dichloromethane were conserved in formaldehyde (6). Bacterial dehalogenases acting on 2-haloalkanoic acids were extensively studied (16). They are not dependent on glutathione but catalyze the direct displacement of halogen with a hydroxyl group from water. In the case of dichloromethane dehalogenase from *Hyphomicrobium* sp. strain DM2, however, nucleophilic substitution is initiated by glutathione. This enzyme, whose properties are described in the present paper, is the first haloalkane dehalogenase of bacterial origin.

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MATERIALS AND METHODS

Chemicals and purification materials. Protein standards were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany. Formaldehyde dehydrogenase and glutathione were purchased from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol (DTT) and all other chemicals were from Fluka, Buchs, Switzerland. DEAE-cellulose (Whatman DE-52) was from Whatman Ltd., Kent, United Kingdom, and hydroxylapatite (Bio-Gel HTP) was from Bio-Rad Laboratories, Richmond, Calif. Sephadex G-200 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. *N*-Pentyl-Sepharose was prepared by the method of Shaltiel (19) by linking 1-aminopentane covalently to cyanogen bromide-activated Sepharose 4B, which was purchased from Pharmacia Fine Chemicals.

Organism and growth conditions. *Hyphomicrobium* sp. strain DM2 (22) was grown at 30°C in continuous culture under conditions of carbon limitation as described by Brunner et al. (3). A 2-liter fermentor (MBR Bioreactor AG, Wetzikon, Switzerland) with a working volume of 1.7 liters was run at a dilution rate of 0.03 h^{-1}. The minimal medium used contained (grams per liter): KH_2PO_4, 1.36; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0. After sterilization the medium was supplemented with the trace elements solution described by Stucki et al. (22), and dichloromethane was added to give a final concentration of 120 mM in the storage vessel. The effluent from the chemostat of a 24-h period was collected in an ice-cooled flask. Cells were harvested by centrifugation, washed once with 50 mM Tris-hydrochloride (pH 7.5), and stored at −20°C until use.

Enzyme assays. Dichloromethane dehalogenase activities were determined by chemically or enzymatically assaying the rate of formaldehyde production. The enzyme assay based on the chemical determination of formaldehyde was a slight modification of the method described by Stucki et al. (22). The standard incubation mixture contained in a total volume of 2.0 ml 100 mM Tris-sulfate (pH 8.0), 5 mM reduced glutathione, 200 μg of pure dichloromethane dehalogenase or an adequate amount of a crude preparation per ml, and 5 mM dichloromethane to start the reaction. Wheaton tubes of 4.0 ml (total volume) closed with Teflon-lined screw

caps to prevent volatilization of dichloromethane were used for incubating the mixture at 30°C. After 5 and 15 min of incubation samples were taken for assaying formaldehyde by the 2,4-pentanedione method as previously described (22). Enzyme activities were calculated from the formaldehyde production rate between 5 and 15 min of incubation.

A coupled assay with NAD-dependent formaldehyde dehydrogenase (EC 1.2.1.1) from *Pseudomonas putida* was used for determining the kinetic parameters of pure dichloromethane dehalogenase. The standard reaction mixture of this assay contained in a total volume of 1.2 ml 50 mM potassium phosphate (pH 8.0), 5 mM reduced glutathione, 0.5 mM NAD, 1.2 mg of formaldehyde dehydrogenase (5.5 mkat/kg of protein), and 12 μg of pure dichloromethane dehalogenase. After preincubation of these components at 30°C for 3 min in a quartz cuvette that was tightly closed with a Teflon stopper, the reaction was started by the addition of dichloromethane to a final concentration of 2 mM. The absorption increase at 339 nm was monitored. Blank reactions with assay mixtures lacking either dichloromethane dehalogenase or formaldehyde dehydrogenase, or both, were not significant. Dehalogenase activity was calculated from the rate of NADH production.

The apparent K_m value of formaldehyde dehydrogenase under the standard conditions of the dehalogenase assay was 75 μM. This value was used to calculate the appropriate amount of formaldehyde dehydrogenase to be included in the coupled assay (18). After a lag time of 1 min the formation of NADH in the coupled assay proceeded linearly.

Determination of dichloromethane dehalogenase activity in a given preparation by the two assay methods described above led to identical results. However, the two methods differed with respect to their sensitivity. The limit of detection for formaldehyde, the product of the enzyme reaction, was 50 nM in the chemical assay and 1 nM in the coupled assay.

The amount of catalytic activity in enzyme preparations is expressed in kat, 1 kat corresponding to the amount of activity catalyzing the transformation of 1 mol of substrate per second.

Enzyme purification. All enzyme purification operations were carried out at 0 to 5°C.

Crude extract. Frozen wet cells (51 g) were thawed in 200 ml of buffer A (50 mM Tris-hydrochloride [pH 7.5], 5 mM DTT, 0.1 mM EDTA, 25% [vol/vol] glycerol). Cell disruption was performed by passing 40-ml portions of the suspension three times through a French pressure cell at 35 MPa. Intact cells and cell debris were removed by centrifugation at 30,000 × *g* for 30 min.

Protamine sulfate treatment. Crude extract (220 ml) was freed from nucleic acids by slowly adding 44 ml of a 2% protamine sulfate solution. After stirring for 30 min the precipitate was removed by centrifugation at 30,000 × *g* for 30 min.

DEAE-cellulose column chromatography. The supernatant solution was diluted with the same volume of a 25% (vol/vol) glycerol solution containing 0.1 mM EDTA, and its pH was adjusted to 8.2 by the addition of KOH. This protein solution was applied to a column (5 by 60 cm) of DEAE-cellulose equilibrated with buffer B (25 mM Tris-hydrochloride [pH 8.2], 2.5 mM DTT, 0.1 mM EDTA, 25% [vol/vol] glycerol). After washing with 1 volume of buffer B, elution was carried out with a linear gradient (4 liters) of 0 to 100 mM potassium phosphate in buffer B at a flow rate of 250 ml/h. Fractions (15 ml) containing dichloromethane dehalogenase activity were pooled. To remove the glycerol which reduces the hydrophobic interactions in the next purification step, the protein solution of the pooled fractions (560 ml) was diluted with 1 volume of 2 M potassium phosphate (pH 7.5) containing 2.5 mM DTT and 0.1 mM EDTA. The volume of the solution was reduced to approximately one-fifth in a hollow-fiber concentrator DC2 (Amicon Corp., Lexington, Mass.). The preparation was then diluted fivefold in buffer C (1 M potassium phosphate [pH 7.5], 2.5 mM DTT, 0.1 mM EDTA) and concentrated again.

***N*-Pentyl-Sepharose column chromatography.** A 265-ml volume of the enzyme solution obtained in the previous step was applied to a column (1.6 by 40 cm, 80-ml bed volume) of *N*-pentyl-Sepharose equilibrated with buffer C at a flow rate of 80 ml/h. After the column was washed with 1 volume of the same buffer, the enzyme was eluted with 0.1 M potassium phosphate (pH 7.5)–2.5 mM DTT–0.1 mM EDTA. Fractions (10 ml) containing enzyme activity were pooled and concentrated by ultrafiltration with an Amicon PM10 membrane to 60 ml. The concentrate was desalted by passage through Sephadex G-25 M equilibrated with buffer D (50 mM potassium phosphate [pH 7.0], 2.5 mM DTT, 0.1 mM EDTA, 25% [vol/vol] glycerol) and concentrated again by ultrafiltration.

Hydroxylapatite column chromatography. A 55-ml volume of concentrated enzyme solution was applied to a hydroxylapatite column (1.6 by 60 cm) equilibrated with buffer D. After washing with 2 volumes of buffer D, a linear gradient (1.0 liters) from 50 to 300 mM potassium phosphate in buffer D was applied. Elution was carried out at a flow rate of 40 ml/h. Fractions (10 ml) containing enzyme activity were pooled, concentrated to 170 ml, and stored in portions at −20°C.

Protein in crude extracts and in the purified enzyme preparations was estimated by the method of Bradford (2) with bovine serum albumin in the appropriate buffers as a standard.

Electrophoresis and MW determination. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed in slab gels (13.0 by 13.0 by 0.15 cm) by the method of Laemmli (11) with separating gels with 10% acrylamide, 0.1% SDS, and Tris buffers or by the method of Weber and Osborn (24) with 5% acrylamide gels, 0.1% SDS, and sodium phosphate buffers. Dichloromethane dehalogenase and reference proteins were denatured by incubation at 95°C for 3 min in a solution containing 1.0% (wt/vol) SDS and 1.0% (vol/vol) 2-mercaptoethanol. Gels were stained for protein with Serva blue G and destained by diffusion.

For estimating the molecular weight (MW) of the dehalogenase subunit, the pure enzyme and suitable standard proteins (20 μg each) were electrophoresed as described above. The mobility of the bands was calculated relative to the tracking dye bromophenol blue.

The MW of the pure enzyme under native conditions was

TABLE 1. Purification of dichloromethane dehalogenase

Enzyme fraction	Vol (ml)	Total protein (g)	Total activity (nkat)	Sp act (mkat/kg)	% Yield	Fold purification
Crude extract	220	5.77	19,900	3.45	100	
Protamine sulfate treatment	275	4.14	21,300	5.15	111	1.5
DEAE-cellulose	560	1.54	18,360	11.9	92	3.4
N-Pentyl-Sepharose	183	0.97	13,660	14.1	69	4.1
Hydroxylapatite	170	0.69	11,970	17.3	60	5.0

determined by gel filtration on a Sephadex G-200 column (2.6 by 100 cm) equilibrated with 0.2 M potassium phosphate (pH 7.0)–0.1 mM EDTA–25% (vol/vol) glycerol. The column was calibrated with the following reference proteins (4.0 mg of each) that were applied in two batches of 1.0 ml each: ferritin, 450,000; catalase, 240,000; aldolase, 158,000; bovine serum albumin, 68,000; ovalbumin, 45,000; chymotrypsinogen, 25,000; cytochrome *c*, 12,500. In a third run 4.0 mg of pure dehalogenase in 1.0 ml of buffer was chromatographed.

Dimethyl suberimidate subunit cross-linking. Covalent cross-linking of enzyme subunits was carried out by the method of Davies and Stark (4). A portion of the pure enzyme preparation (Table 1) was freed of minor degradation products by precipitation in $(NH_4)_2SO_4$ of 55% saturation and overnight dialysis against 50 mM potassium phosphate (pH 7.5). A 40-μl volume of this preparation (80 μg of enzyme) was mixed with 40 μl of 0.4 M triethanolamine (pH 8.5) containing 800 μg of dimethyl suberimidate and incubated at 37°C for 2 h. The reaction was stopped by adding SDS and 2-mercaptoethanol to a final concentration of 1% each, followed by 3 min of incubation at 95°C. Bromophenol blue solution (5 μl) serving as a front marker was added to all samples before they were subjected to SDS-PAGE on 5% gels by the method of Weber and Osborn (24). Catalase (monomer, MW, 60,000), bovine serum albumin (MW, 68,000), and rabbit muscle aldolase (monomer, MW, 39,500; dimer, MW, 79,000; trimer, MW, 118,500; tetramer, MW, 158,000) that were cross-linked as described by Davies and Stark (4) were used as marker proteins to establish a calibration curve for MW estimation of the different dehalogenase oligomers.

RESULTS

Purification of dichloromethane dehalogenase. Dichloromethane dehalogenase from cells grown on dichloromethane as the only carbon and energy source was purified fivefold with 60% yield (Table 1). DEAE-cellulose chromatography, the first chromatographic step in the purification procedure, resulted in a clear separation of the enzyme from most of the other cellular proteins. Since the enzyme was unstable in Tris buffers of low molarity but retained its activity in high-ionic-strength phosphate buffers, hydrophobic column chromatography was chosen as the next purification step. Adsorption of the enzyme to *N*-butyl-Sepharose was incomplete, whereas the strong interaction of the protein with *N*-hexyl-Sepharose hindered its elution by aqueous buffer solutions. *N*-Pentyl-Sepharose, however, exhibited suitable properties. Adsorption of dichloromethane dehalogenase to this matrix occurred in 1 M potassium phosphate, and the enzyme was desorbed completely by elution of the column with a 0.1 M solution of the same buffer. After a final step of hydroxylapatite chromatography a purified protein preparation was obtained which still contained minor contaminants (Fig. 1). Since the proportion of these impurities increased upon storage, they probably were degradation products of the dichloromethane dehalogenase. Precipitation of the putative degradation products in $(NH_4)_2SO_4$ of 55% saturation led to an electrophoretically homogeneous preparation of dichloromethane dehalogenase that was judged to be more than 98% pure (Fig. 1). To confirm that the dehalogenase activity of the purified preparation was due to the protein giving a single band in SDS-PAGE and not to a minor contaminant, the following experiment was carried out. Purified enzyme was electrophoresed under nondenaturing conditions and stained for dehalogenase activity by the activity staining procedure described by Hardman and Slater (8). The stained gel segment was cut out, treated with 1.0% (wt/vol) SDS–1.0% (vol/vol) 2-mercaptoethanol, inserted into the slot of a second slab gel, and electrophoresed under denaturing conditions. Staining for protein gave a single band whose mobility was identical to the pure enzyme subjected directly to SDS-PAGE. The pure enzyme was stored at a concentration of 4 mg/ml in 0.1 M potassium phosphate (pH 7.5)–2.5 mM DTT–0.1 mM EDTA–25% (vol/vol) glycerol at −20°C. Under these conditions it retained full activity for at least 6 months. Within 6 days of storage at room temperature the preparation had lost 50% of its initial activity.

TABLE 2. Apparent K_m values for dihalomethanes and glutathione

Substrate or cofactor	Apparent K_m (μM)	V_{max} (mkat/kg of protein)
CH_2Cl_2	30	16.9
CH_2BrCl	15	15.3
CH_2Br_2	13	15.8
CH_2I_2	5	4.1
GSH	320	

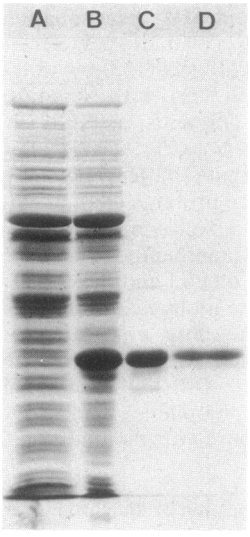

FIG. 1. SDS-PAGE of dichloromethane dehalogenase of *Hyphomicrobium* sp. strain DM2. Protein samples were electrophoresed under denaturing conditions by the method of Laemmli (11). (A) Protein (120 μg) of a crude extract of methanol-grown cells; (B) protein (150 μg) of a crude extract of dichloromethane-grown cells; (C) dichloromethane dehalogenase (20 μg) after chromatography on hydroxylapatite; (D) dichloromethane dehalogenase (20 μg) in the supernatant after precipitation with ammonium sulfate of 55% saturation.

Since it was possible to obtain a homogeneous dehalogenase preparation by a fivefold enrichment in a purification procedure reducing the total amount of protein by a factor of 8.3, the enzyme must represent between 12 and 20% of the total soluble protein of dichloromethane-grown cells. The electrophoretic pattern obtained by SDS-PAGE of a crude extract of such cells indeed illustrates that the most prominent band is due to dichloromethane dehalogenase (Fig. 1). From a comparison of this pattern with the pattern of methanol-grown cells (Fig. 1) it is also evident that dichlo-

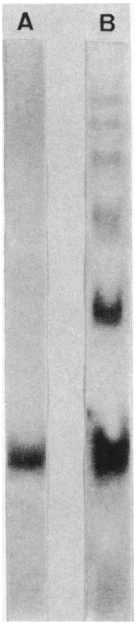

FIG. 2. SDS-PAGE after subunit cross-linking with dimethyl suberimidate. SDS-PAGE was carried out by the method of Weber and Osborn (24) in a slab gel containing 5% acrylamide. (A) Dichloromethane dehalogenase monomer; (B) cross-linked dichloromethane dehalogenase.

romethane dehalogenase is the only protein that is highly induced during growth on the xenobiotic substrate.

Molecular characteristics of the purified enzyme. The MW of the pure enzyme under native conditions was determined at 195,000 ± 10,000 by gel filtration on Sephadex G-200.

SDS-PAGE of purified dichloromethane dehalogenase gave a single band with an estimated MW of 33,000 ± 1,000, thus suggesting a hexameric tertiary structure of the enzyme. The subunits of the pure enzyme under native conditions were cross-linked to a limited extent by the bifunctional reagent dimethyl suberimidate. SDS treatment of such a preparation and subsequent separation of its components by SDS-PAGE led to the appearance of six bands (Fig. 2). Their MWs corresponded to the MWs of the different oligomers of dichloromethane dehalogenase (monomer to hexamer). This result thus confirmed that dichloromethane dehalogenase is a hexamer composed of identical subunits.

Kinetic properties and substrate specificity. The Michaelis constants for the four dihalomethanes (Table 2) were determined with purified enzyme by the coupled enzyme assay. Saturation curves for the dihalomethanes and for glutathione were hyperbolic. The linearization method of Hanes (7) was used to calculate apparent K_m and V_{max} values. To reach half-maximal velocity the enzyme required a concentration of glutathione that was about 10 times higher than the concentration of dichloromethane. When substrate inhibition by dichloromethane was investigated it was found that the enzyme was slightly inhibited at dichloromethane concentrations exceeding 5 mM. At 50 mM dichloromethane the enzyme still exhibited an activity corresponding to 70% of V_{max}.

A distinct activity optimum of the purified enzyme at pH 8.5 was found. It was determined with the assay conditions and buffers described by Stucki et al. (22). These authors determined an activity optimum of dichloromethane dehalogenase at pH 8.0 in crude extracts of the same organism. In accordance with their observations on crude enzyme preparations glutathione could not be substituted as a cofactor of the pure enzyme by other sulfhydryl agents such as 2-mercaptoethanol, DTT, or cysteine.

Testing halogenated aliphatic hydrocarbons other than dihalomethanes as substrates of the pure enzyme met with experimental difficulties. Assays based on conventional determinations of liberated halide ions were not sensitive enough; tests based on substrate disappearance also were not sensitive enough. Solutions (2 mM) of a number of potential substrates therefore were incubated for 50 h under standard conditions with pure enzyme and subsequently assayed for the presence of free chloride by X-ray fluorescence spectrometry. The substances tested were 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, tetrachloroethane, 1,1-dichloroethene, trichloroethene, tetrachloroethene, 1-chloropropane, and chlorobenzene. 1,2-Dichloroethane was the only compound that was dehalogenated by pure dichloromethane dehalogenase. The reaction rate of the enzyme with this substrate, however, was approximately 1,000 times lower than the reaction rate with dichloromethane.

Competitive inhibition by substrate analogs. The inhibitory action of a number of chlorinated aliphatic compounds on the dehalogenation of dichloromethane was investigated. Table 3 lists those compounds that exhibited an inhibitory effect. Inhibition by these chemicals was reversible since removal of the inhibitors by gel filtration led to restoration of the initial specific activity of the enzyme. It appears that biterminally monochlorinated alkanes were the most potent inhibitors, followed by the monoterminally monosubstituted chloroalkanes. The extent of inhibition decreased as the chain length of the putative substrate analogs increased. Compounds without an inhibitory effect were trichloromethane, tetrachloromethane, 1,2,2,2-tetrachloroethane, 1,1-dichloroethene, trichloroethene, and tetrachloroethene.

A strong and a moderate inhibitor, 1,2-dichloroethane and

TABLE 3. Inhibition of pure dichloromethane dehalogenase by chlorinated compounds

	Inhibitor[a]	% Inhibition[b]
Cl_2CHCH_3	1,1-Dichloroethane	90
$Cl_2CHCH_2OCH_3$	2,2-Dichloroethyl methyl ether	38
$Cl_2CHCOOH$	Dichloroacetic acid	31
$Cl_2CHCOOCH_3$	Methyl dichloroacetate	33
Cl_2CHCH_2Cl	1,1,2-Trichloroethane	15
$Cl_2CHCHCl_2$	1,1,2,2-Tetrachloroethane	0
$ClCH_2CH_2Cl$	1,2-Dichloroethane	100
$Cl(CH_2)_3Cl$	1,3-Dichloropropane	94
$Cl(CH_2)_4Cl$	1,4-Dichlorobutane	75
$ClCH_2CH_2OCH_3$	2-Chloroethyl methyl ether	48
$Cl\diagdown CH = CH\diagdown Cl$	*trans*-1,2-Dichloroethylene	43
$Cl\diagdown CH = CH\diagup Cl$	*cis*-1,2-Dichloroethylene	20
$ClCH_2CH_2CH_3$	1-Chloropropane	87
$Cl(CH_2)_3CH_3$	1-Chlorobutane	72
$Cl(CH_2)_4CH_3$	1-Chloropentane	50
$Cl(C_6H_5)$	Chlorobenzene	24

[a] Inhibitors were present at a final concentration of 1 mM in the assay mixture for the coupled enzyme assay with 0.05 mM dichloromethane. It was verified that the coupling enzyme (formaldehyde dehydrogenase) was not affected by the inhibitors.

[b] Inhibition is expressed as the percentage of activity decrease relative to the activity of the noninhibited control.

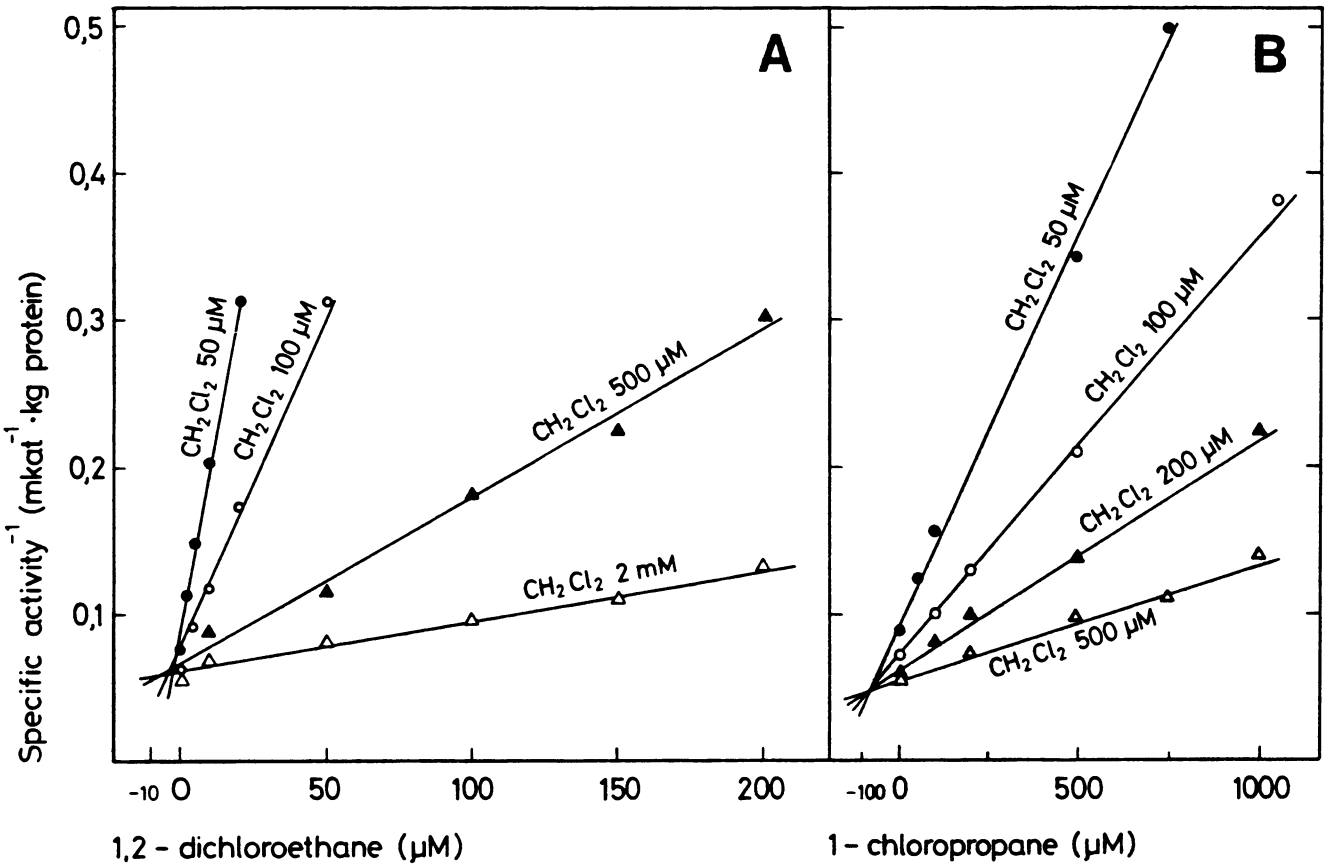

FIG. 3. Competitive inhibition of pure dichloromethane dehalogenase by 1,2-dichloromethane (A) and 1-chloropropane (B).

1-chloropropane, respectively, were used to identify the type of inhibition and to determine the K_i values by the method of Dixon (5; Fig. 3). Replots of the slopes of the Dixon plots versus the reciprocal of the corresponding substrate concentrations ($[CH_2Cl_2]^{-1}$) gave straight lines through the origin, indicating that at least in these two cases inhibition was of the competitive type. K_i values of 3 and 56 µM were found for 1,2-dichloroethane and 1-chloropropane, respectively.

DISCUSSION

Dichloromethane represents a novel substrate for methylotrophic growth that has been introduced into the environment by the industrial activity of humans (14). *Hyphomicrobium* sp. strain DM2, one of the bacteria using this xenobiotic substrate as the sole carbon and energy source, possesses the enzyme dichloromethane dehalogenase for catalyzing the first step in the dissimilation of dichloromethane. This enzyme is highly inducible by its substrate and constitutes about 16% of the total soluble protein of induced cells. It has an extremely low turnover number of 33 mol of dichloromethane per mol of enzyme subunit per min. Since an apparent K_m value for dichloromethane of 30 µM was measured, the low efficiency of the overall enzymatic process is not due to incompetence in substrate binding but rather to a low reaction rate. An even higher affinity of the enzyme was observed for dibromomethane and diiodomethane, compounds that exhibited apparent Michaelis constants of 13 and 5 µM, respectively. Since the distance between carbon and halogen atoms increases in the order C-Cl, C-Br, C-I, the binding site of dichloromethane dehalogenase seems to be optimally adapted to accommodate diiodomethane, the bulkiest molecule among the dihalomethanes.

In *Hyphomicrobium* sp. strain DM2 growing on dichloromethane the low specific activity of pure dichloromethane dehalogenase is compensated by the high intracellular concentration of the enzyme. This strategy of the cell may indicate that dichloromethane dehalogenase represents a recently evolved enzyme whose catalytic activity is still in the process of being optimized.

The transformation catalyzed by dichloromethane dehalogenase bears some resemblance to the reactions of 2-haloalkanoic acid dehalogenases (EC 3.8.1.2 and EC 3.8.1.3) and glutathione *S*-transferases (EC 2.5.1.18). In the case of the 2-haloalkanoic acid dehalogenases this similarity is restricted to the ability to cleave carbon-halogen bonds of aliphatic molecules. There are no halogenated compounds known that serve as substrates for both types of enzymes. Also the reaction mechanisms seem to be different, requiring reduced glutathione in one case but being independent of a cofactor in the other. Furthermore, dichloromethane dehalogenase is a hexameric protein with a MW of 195,000, whereas the five different 2-haloalkanoic acid dehalogenases that have been purified so far are either monomers or dimers with a MW of 60,000 for the largest of these enzymes (16). Dichloromethane dehalogenase thus differs in its structure from the known dehalogenases and exhibits novel catalytic properties in that it represents the first enzyme acting on haloalkanes.

The reaction mechanism of dichloromethane dehalogenase is similar to the reaction mechanism of glutathione *S*-transferases. In higher organisms these enzymes play an important role in detoxifying xenobiotic compounds. They catalyze the nucleophilic attack on electrophiles to produce thioether derivatives referred to as glutathione conjugates (9). Recently, several bacterial glutathione *S*-transferases of unknown function were detected in crude extracts on the basis of their activity towards 1-chloro-2,4-dinitrobenzene (13, 15, 20, 23). Although this compound is reported to be a substrate for all glutathione *S*-transferases that have been purified so far (9), purified dichloromethane dehalogenase did not react with 1-chloro-2,4-dinitrobenzene (D. Kohler-Staub, unpublished data). Quite in contrast to the wide substrate spectrum of the glutathione *S*-transferases from mammalian sources, effective dehalogenation by dichloromethane dehalogenase was restricted to dihalomethanes with a faint activity towards 1,2-dichloroethane. Since none of the bacterial glutathione *S*-transferases have been purified so far, their molecular structure and substrate range cannot be compared to the properties of dichloromethane dehalogenase from *Hyphomicrobium* sp. strain DM2. Evidence for or against an evolutionary relationship between this enzyme and the bacterial glutathione *S*-transferases thus is not available.

ACKNOWLEDGMENTS

This work was supported by a grant from the Swiss Federal Institute of Technology, Zürich, Switzerland, and by Ciba-Geigy AG, Basel, Switzerland.

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