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Purification and Characterization of 2,4,6-Trichlorophenol-4-Monooxygenase, a Dehalogenating Enzyme from *Azotobacter* sp. Strain GP1

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The enzyme which catalyzes the dehalogenation of 2,4,6-trichlorophenol (TCP) was purified to apparent homogeneity from an extract of TCP-induced cells of *Azotobacter* sp. strain GP1. The initial step of TCP degradation in this bacterium is inducible by TCP; no activity was found in succinate-grown cells or in phenol-induced cells. NADH, flavin adenine dinucleotide, and O_2 are required as cofactors. As reaction products, 2,6-dichlorohydroquinone and Cl^- ions were identified. Studies of the stoichiometry revealed the consumption of 2 mol of NADH plus 1 mol of O_2 per mol of TCP and the formation of 1 mol of Cl^- ions. No evidence for membrane association or for a multicomponent system was obtained. Molecular masses of 240 kDa for the native enzyme and 60 kDa for the subunit were determined, indicating a homotetrameric structure. Cross-linking studies with dimethylsuberimidate were consistent with this finding. TCP was the best substrate for 2,4,6-trichlorophenol-4-monooxygenase (TCP-4-monooxygenase). The majority of other chlorophenols converted by the enzyme bear a chloro substituent in the 4-position. 2,6-Dichlorophenol, also accepted as a substrate, was hydroxylated in the 4-position to 2,6-dichlorohydroquinone in a nondehalogenating reaction. NADH and O_2 were consumed by the pure enzyme also in the absence of TCP with simultaneous production of H_2O_2. The NH_2-terminal amino acid sequence of TCP-4-monooxygenase from *Azotobacter* sp. strain GP1 revealed complete identity with the nucleotide-derived sequence from the analogous enzyme from *Pseudomonas pickettii* and a high degree of homology with two nondehalogenating monooxygenases. The similarity in enzyme properties and the possible evolutionary relatedness of dehalogenating and nondehalogenating monooxygenases are discussed.

Polychlorinated phenols are widespread environmental pollutants, and their degradation has been the subject of numerous investigations (for reviews, see references 5, 9, 10, and 21). While many different microorganisms with the capacity to degrade these compounds have been isolated, relatively few reports have dealt with the initial step of their degradation. Moreover, the majority of these investigations were performed with whole cells, crude extracts, or partially purified enzymes, and so far only two polychlorophenol-dehalogenating enzymes from *Burkholderia* (formerly *Pseudomonas*) *cepacia* AC1100 (33) and from a *Flavobacterium* sp. (34) have been purified to homogeneity. Other polychlorophenol dehalogenases were partially purified from an *Arthrobacter* sp. (23) or found to be membrane-associated enzymes in *Rhodococcus chlorophenolicus* (26, 27) and *Mycobacterium fortuitum* (28).

The dehalogenation of 2,4,6-trichlorophenol (TCP) was detected in crude extracts of *Streptomyces rochei* 303 (7) and *Pseudomonas pickettii* (12). The conversion of TCP to 2,6-dichlorohydroquinone was also observed with whole cells from *Pseudomonas cepacia* AC1100 (25).

In this paper, we describe the purification and characterization of a TCP-dehalogenating enzyme from the TCP-degrading soil bacterium *Azotobacter* sp. strain GP1.

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MATERIALS AND METHODS

Organism and growth conditions. *Azotobacter* sp. strain GP1 is deposited under accession no. DSM 6428 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany, and under ATCC 49806 at the American Type Culture Collection, Rockville, Md.

The biomass was obtained from a 100-liter fermentor containing a succinate (4-g/liter) mineral salts medium with a composition as described previously (13) and inoculated with 2 liters of a preculture in the same medium. At the end of the logarithmic growth phase, cells were induced by three subsequent additions of TCP (50, 100, and 150 mg/liter). Cells were harvested by centrifugation before the third portion of TCP was completely degraded.

Cells which were noninduced and phenol induced (with 50 mg of phenol per liter) were grown in a 20-liter fermentor.

Chemicals. 2,6-Dichlorohydroquinone was a gift from L. A. Golovleva, Pushchino, Russia. TCP and 2,3-, 2,4-, and 3,4-dichlorophenols were purchased from Merck, Darmstadt, Germany. Pentachlorophenol (PCP) and 2,3,4,5- and 2,3,5,6-tetrachlorophenols were purchased from Aldrich-Chemie, Steinheim, Germany. All other chlorophenols, flavin adenine dinucleotide (FAD), and flavin mononucleotide were from Fluka, Buchs, Switzerland. 2,6-Dichlorobenzoquinone was from Eastman Kodak Co., Rochester, N.Y. NADH, NADPH, ATP, ADP, coenzyme A, and all inhibitors tested in this study were obtained from Sigma, Deisenhofen, Germany.

Buffers. Many purification steps and enzyme tests were performed with 25 mM Tris-HCl buffer, pH 7.0 (buffer A). The pH dependence of the enzyme reaction was determined by using the following buffers (25 mM): Britton-Robinson (composed of acetic acid, H_3PO_4, H_3BO_3, and NaOH) from pH 5.0 to 11.5; Davies (citric acid, KH_2PO_4, $Na_2B_4O_7 \cdot 10H_2O$, Tris, KCl, HCl, and NaOH) from pH 6.0 to 8.5; citrate-phosphate buffer from pH 5.0 to 7.8; KH_2PO_4-Na_2HPO_4 buffer from pH 5.0 to 8.0; and Tris-HCl from pH 7.0 to 9.0.

Preparation of crude extract and membrane proteins. Frozen cells (40 g [wet weight]) were suspended in 80 ml of buffer A and disrupted by a 20-min sonication with a Branson model 450 ultrasonic disintegrator. Cell debris and intact cells were removed by centrifugation at 48,000 × *g* for 60 min at 4°C. The supernatant (100 ml), designated crude extract, was centrifuged in an L5-65 ultracentrifuge (Beckman Instruments, Munich, Germany) 150,000 × *g* at 4°C for 2 h. The pellet was treated with Triton X-100 (0.1% final concentration) for the solubilization of membrane proteins. Both the solubilized membrane proteins and the supernatant (cytoplasmatic proteins) were examined for dehalogenase activity by the high-pressure liquid chromatography (HPLC) test. By testing

an active enzyme preparation in the presence of the corresponding concentration of Triton X-100, it was shown that the detergent did not influence the enzyme activity.

Enzyme assays. The following tests were developed to determine TCP-dehalogenating activity. For routine investigations, the spectrophotometric NADH test and the HPLC test were applied. One unit of enzyme activity was defined as the amount of enzyme catalyzing the conversion of 1 μmol of TCP per min.

(i) HPLC test. The decrease of TCP concentration in the course of the enzyme reaction was monitored by HPLC analysis. From the reaction mixture, containing 0.05 to 0.35 mM TCP, 5 mM NADH, 0.03 mM FAD, and 0.5 to 10 mg of protein per ml in buffer A, aliquots of 0.3 ml were withdrawn after different incubation times. To these samples, 0.1 ml of 1 N H_2SO_4 was added, and precipitated protein was removed by centrifugation. The supernatant was neutralized with 1 N NaOH, and after a further centrifugation step the supernatant was subjected to HPLC analysis.

(ii) NADH test. TCP-specific NADH oxidation was initiated by the addition of the chlorophenol (0.1 mM) to a cuvette containing 0.8 ml of a mixture of 0.03 mM FAD, 0.15 mM NADH, and 0.05 to 1 mg of protein per ml in buffer A. NADH oxidation in the absence of TCP was subtracted from the rate observed in the presence of TCP. The oxidation rate was calculated by using a molar absorption coefficient of 6,300 M^{-1} cm^{-1} for NADH at 340 nm.

(iii) O_2 test. Under the same reaction conditions as described for the NADH test, TCP-specific O_2 consumption was determined polarographically with the aid of a Clark DW1 oxygen electrode (Bachofer, Reutlingen, Germany) with stirring in a gastight 0.8-ml reaction vessel.

Reaction stoichiometry. The molar ratio of NADH and O_2 consumption was obtained by using an excess of TCP (0.1 mM), and for the chloride/TCP ratio a reaction-limiting amount of TCP and a surplus of NADH (5 mM) were applied. The conversion of NADH and O_2 was measured as described above, and the TCP conversion was monitored spectrophotometrically at 311 nm. The chloride contents of samples from the HPLC test were determined by the $Hg(SCN)_2$ method (6) with a chloride-free phosphate buffer (25 mM, pH 7.0).

Enzyme purification. All purification steps were performed at 4°C.

(i) Anion-exchange chromatography on DEAE-cellulose. A 100-ml volume of crude extract obtained from 40 g of *Azotobacter* cells was applied to a DE52 cellulose anion-exchange column (bed volume, 90 ml) previously equilibrated with buffer A. After the column was washed with 1,000 ml of the same buffer, the dehalogenase was eluted with 600 ml of a linear concentration gradient from 0 to 0.5 M KCl. Activity was eluted at 0.2 M KCl as a single, symmetrical peak.

(ii) Metal-chelating affinity chromatography. Active fractions from DE52 cellulose anion-exchange chromatography (50 ml) were diluted with 400 ml of a 50 mM Tris-acetate buffer (pH 8.0) containing 0.5 M NaCl. The solution was concentrated to 50 ml in an Amicon ultrafiltration cell equipped with a YM100 filter. The concentrated protein was applied to a metal-chelating chromatography column (with 50 ml of chelating Sepharose) which had been previously loaded with 200 ml of an aqueous nickel sulfate solution (5 g/liter), and equilibrated with 50 mM Tris-acetate buffer (pH 8.0) plus 0.5 M NaCl. Unbound protein was washed off with the same equilibration buffer, and elution was performed with 400 ml of a linear gradient of 0 to 0.2 mM imidazole in equilibration buffer. The enzyme was eluted as a single peak at 0.1 mM imidazole.

(iii) Resource Q anion-exchange chromatography. After the removal of imidazole and concentration to 5 ml by ultrafiltration, the enzyme solution was applied to a Resource Q anion-exchange column (6 ml) from Pharmacia, Uppsala, Sweden, previously equilibrated with buffer A. Elution of proteins was achieved with a Pharmacia fast protein liquid chromatography system. After a washing with 20 ml of buffer A, the enzyme eluted at 0.25 M KCl as a single peak in a linear concentration gradient from 0 to 0.4 M KCl in buffer A (70 ml). Active fractions (5 ml) were concentrated to 1 ml by ultrafiltration and stored in small portions at −20°C.

Analytical methods. UV spectra were recorded with a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer, and spectrophotometric tests were done with a Zeiss PMQ-3 spectrometer equipped with a Linseis LS 52-2 recorder.

HPLC analysis was performed with a Nucleosil 100 RP 18 column (particle size, 5 μm). Eluents were 5 mM KH_2PO_4, adjusted to pH 2 with 1 N sulfuric acid-methanol (30/70, vol/vol) (eluent 1); 5 mM KH_2PO_4 (pH 2)–methanol (50/50, vol/vol) (eluent 2); and an acetonitrile–3 mM phosphoric acid gradient with a linear increase of acetonitrile concentration from 30 to 100% in 25 min (flow rate, 1 ml per min) (eluent 3). For eluents 1 and 2, a flow rate of 0.7 ml per min was applied. The elution of substrates and products was monitored with an optical scanning detector from TSP, Darmstadt, Germany. Spectra of the separated compounds were recorded from 200 to 400 nm.

The protein concentration was determined by the method of Bradford (4) with bovine serum albumin as the standard. During enzyme separation on Resource Q, Superose 12, and Superdex G200, protein content was recorded at 280 nm with a Pharmacia UV-2 monitor.

Enzyme purity and the molecular weights of the subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (22) with 10% gels with low-molecular-weight standard proteins (M_rs of 14,400 to 94,000) from Pharmacia. The proteins were stained with Coomassie blue R250. The M_r was calculated from a standard linear regression curve of reference proteins.

The molecular weight of the native protein was estimated by fast protein liquid chromatography gel filtration with Superose 12 and Superdex G200 (high load 16/60), both from Pharmacia, with nine standard proteins (M_rs of 69,000 to 440,000) from Boehringer, Mannheim, Germany. Native molecular weight was also obtained from sedimentation analysis with a Beckman model E analytical ultracentrifuge at 56,000 rpm with 0.5 ml of a pure enzyme sample in buffer A.

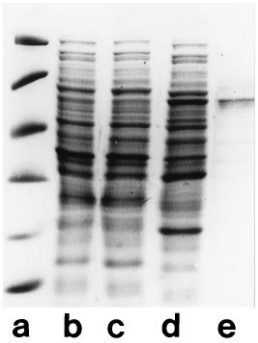

FIG. 1. Induction of TCP-4-monooxygenase from *Azotobacter* sp. strain GP1 by TCP. SDS-PAGE results show the following: lane a, marker proteins of 94, 67, 43, 30, 20.1, and 14.4 kDa; lane b, crude extract from uninduced (succinate-grown) cells; lane c, crude extract from phenol-induced cells; lane d, crude extract from TCP-induced cells; and lane e, purified TCP-4-monooxygenase (1 μg).

The enzyme was stained after protein separation by native 7.5% polyacrylamide gel electrophoresis. One half of the gel was stained with Coomassie blue R250, and the other half was incubated for 10 min in buffer A containing NADH (2 mM) and FAD (0.03 mM) and then for another 10 min in TCP (0.1 mM). Conversion of NADH was visualized under 366-nm UV light as a dark band against a fluorescent background. A gel without addition of TCP served as a control. By incubating the gel in the same mixture as described above but with the addition of 0.2% of 2-(4-iodophenol)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), the enzyme appeared as a red band against a faint yellow background.

Analytical isoelectric focusing was performed in rehydrated gels with a broad-pI calibration kit (pH 3 to 10) from Pharmacia.

The purified 2,4,6-trichlorophenol-4-monooxygenase (TCP-4-monooxygenase) (0.8 and 1.3 mg/ml) was subjected to cross-linking by overnight incubation with dimethylsuberimidate (3.2 and 4.1 mg/ml) in 0.2 M triethanolamine-HCl, pH 8.5. After being washed with 10 mM triethanolamine (pH 8.5), the different samples and the control without cross-linking agent were incubated for 2 h at 37°C in SDS denaturation buffer prior to SDS-PAGE.

The amino-terminal amino acid sequence of the purified enzyme was obtained by automated Edman degradation on an Applied Biosystems model 494 protein sequencer. For the removal of Tris buffer, the enzyme was previously subjected to gel chromatography.

Determination of kinetic parameters. Kinetic parameters were determined by using purified enzyme and monitoring the consumption of NADH as described above. The kinetic values for TCP were obtained with a constant NADH concentration of 0.15 mM and TCP concentrations from 0.5 to 200 μM, and values for NADH were obtained with a constant fixed TCP concentration of 0.1 mM and NADH concentrations from 0.02 to 0.35 mM. K_m and V_{max} were calculated from Eadie-Hofstee and Lineweaver-Burk plots.

RESULTS

Cell growth and enzyme induction. Succinate-grown cells were induced by repeated additions of increasing amounts of TCP, and about 300 to 400 g (wet weight) of *Azotobacter* cells with specific activities of the TCP-dehalogenating enzyme of 0.15 to 0.4 mU per mg of protein was obtained.

No TCP-dehalogenating activity was detected in the crude extract of uninduced and phenol-induced cells. The same results were observed when fractions from the first chromatographic purification step were tested. Obviously, the TCP-converting enzyme is induced by its substrate.

Only the crude extract from TCP-induced cells showed an intense band of the TCP-dehalogenating enzyme in SDS-PAGE (Fig. 1). Remarkably, the extract from TCP-induced cells also indicates the increased expression of a second protein species of about 22 kDa (Fig. 1, lane d).

TABLE 1. Purification of TCP-4-monooxygenase

Purification step	Total protein (mg)	Total activity[a] (mU)	Sp act[a] (mU/mg)	Purification	Recovery (%)
Crude extract	3,359.6	604.7	0.18	1	100
DEAE-cellulose	548.3	806.0	1.47	8.2	133
Ni^{2+}-chelating Sepharose	153.1	561.8	3.67	20.4	93
Resource Q	31.9	219.2	6.87	38.2	36

[a] Activity was determined from TCP conversion in the HPLC test (see Materials and Methods).

Cofactor requirement. For the pure enzyme, the activity with NADH was about 20-fold higher than with NADPH. This preference for NADH was less pronounced in the crude extract (1.5-fold), probably due to the presence of a pyridine nucleotide transhydrogenase.

Fe^{2+} (0.2 mM), Mg^{2+} (0.8 mM), ATP (0.01 to 1 mM), ADP (0.01 to 0.1 mM), and coenzyme A (0.5 mM) did not enhance activity of the purified enzyme.

During purification, a decrease in the yellow color of the protein solution was accompanied by a considerable loss of enzyme activity. Activity could be restored by addition of FAD: 0.03 mM FAD led to a four- to fivefold activation of the DEAE-cellulose-purified enzyme. FAD could not be replaced by flavin mononucleotide or riboflavin. Probably due to a loose binding, FAD was lost during purification and the pure enzyme did not show the typical absorption spectrum of a flavoenzyme with extinction maxima in the visible part of the spectrum. The purified enzyme was reconstituted by addition of 0.03 mM FAD to the enzyme solution. A preincubation was found unnecessary to retain full activity.

TCP was not converted by the enzyme under anaerobic conditions. In a gastight cuvette under nitrogen, the addition of TCP had no effect on NADH consumption. In the presence of O_2, however, TCP addition led to a significant increase of NADH consumption. Similarly, O_2 consumption in the presence of NADH was stimulated by TCP addition.

Due to its cofactor requirement, the TCP-dehalogenating enzyme should be named TCP-4-monooxygenase (30).

Purification of TCP-4-monooxygenase. For the preparation of crude extract, TCP-induced cells were disrupted by ultrasonication, yielding a higher specific activity than French press disintegration. Purification was performed as described in Materials and Methods. Chromatography of the crude extract on DEAE-cellulose was the first purification step. The increase in activity is probably due to the removal of inhibitors. After another two steps and a 38-fold enrichment, an electrophoretically homogeneous enzyme was obtained with a yield of 36% (Table 1). The enzyme from the Resource Q step eluted in gel chromatography on Superose 12 as a single, symmetrical peak, indicating a homogeneous protein. Because gel filtration led to a large loss of activity, it was not applied for routine purification.

The purification of TCP-4-monooxygenase was followed by SDS-PAGE (Fig. 2). Activity staining of a native polyacrylamide gel showed the consumption of NADH and TCP by the TCP-4-monooxygenase. At the position where the enzyme had migrated in the gel, the disappearance of NADH fluorescence was observed under UV light. The effect was enhanced in the presence of TCP. Similarly, the formation of a red formazan, produced from colorless INT, was found at the position of the purified enzyme, identifying the electrophoretically homogeneous protein as TCP-4-monooxygenase.

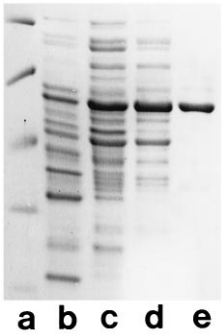

FIG. 2. Purification of TCP-4-monooxygenase from *Azotobacter* sp. strain GP1 as shown by SDS-PAGE. Lane a, marker proteins of 94, 67, 43, 30, and 20.1 kDa; lane b, crude extract; lane c, results after chromatography on DEAE-cellulose; lane d, results after chromatography on Ni^{2+}-chelating Sepharose; lane e, results after chromatography on Resource Q.

Stoichiometry of enzyme reaction and reaction product. The consumption of 2.2 mol of NADH and of 1.1 mol of O_2 together with the formation of 1.3 mol of Cl^- ions per mol of TCP was observed in the reaction catalyzed by TCP-4-monooxygenase (averages of three and two independent experiments), indicating a molar ratio for TCP:NADH:O_2:Cl^- of 1:2:1:1. As also shown in Fig. 3, approximately 1 mol of Cl^- ions was produced from 1 mol of the chlorophenol. 2,6-Dichlorohydroquinone was identified as the TCP dehalogenation product by HPLC analysis (Fig. 4).

Substrate specificity. From all of the phenolic compounds that were tested as potential substrates for TCP-4-monooxygenase, the 2,4,6-trichloro isomer showed the highest conversion rate (Table 2). 2,4,6-Tribromophenol, which like the analogous chloro compound supports growth of *Azotobacter* sp. strain GP1, also served as a good substrate for the dehalogenating enzyme. The majority of the other chlorophenols, which were utilized as substrates by the TCP-4-monooxygenase, bear a chloro substituent in the 4-position, like TCP. 2,6-Dichlorophenol, without a 4-chloro substituent, was found by HPLC analysis to be converted to the same reaction product as TCP, namely, 2,6-dichlorohydroquinone.

The discrepancy between chlorophenol conversion and NADH consumption that was found for 4-chlorophenol, 2,6-

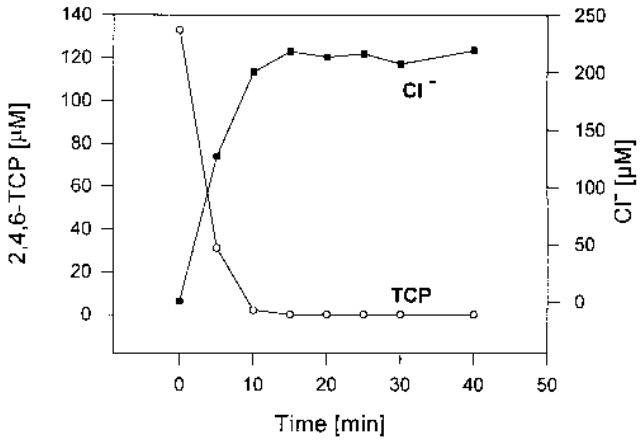

FIG. 3. Release of chloride ions during the conversion of TCP with a pure sample of TCP-4-monooxygenase from *Azotobacter* sp. strain GP1. The concentration of TCP was determined by HPLC analysis and chloride was measured by the $Hg(SCN)_2$ method, as described in Materials and Methods.

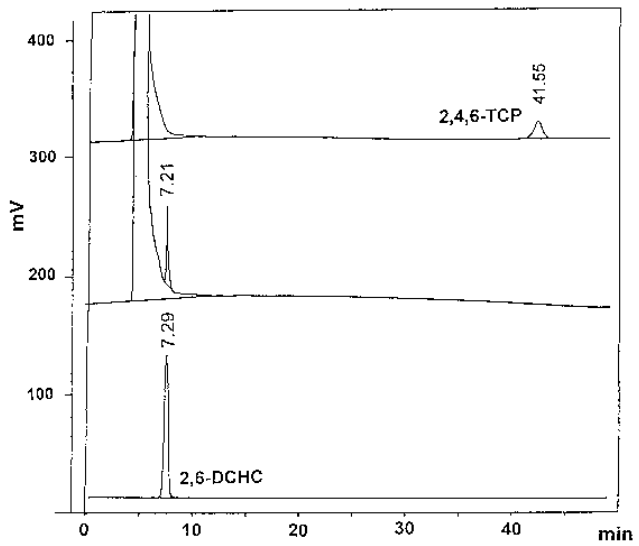

FIG. 4. HPLC analysis of product formation during the conversion of TCP with a pure sample of TCP-4-monooxygenase from *Azotobacter* sp. strain GP1. Top chromatogram, enzyme incubation mixture immediately after addition of TCP; middle, enzyme mixture after 30 min of incubation; bottom, authentic sample of 2,6-dichlorohydroquinone (2,6-DCHC). For the composition of the enzyme incubation mixture and HPLC conditions, see Materials and Methods ("HPLC test").

TABLE 2. Substrate specificity of TCP-4-monooxygenase[a]

Substrate	R_t in HPLC (min)	Relative activity (%)	
		Consumption of substrate[b]	Consumption of NADH[c]
TCP	17.0	100	100
Phenol	ND	ND	0
2-CP	7.1	25	8
3-CP	7.5	0	0
4-CP	7.4	32	0
2,3-DCP	9.1	0	0
2,4-DCP	11.2	80	52
2,5-DCP	9.9	0	0
2,6-DCP	9.0	67	16
3,4-DCP	10.2	50	41
3,5-DCP	13.7	0	0
2,3,4-Trichlorophenol	15.5	0	0
2,3,5-Trichlorophenol	19.5	0	0[d]
2,3,6-Trichlorophenol	12.5	0	0
2,4,5-Trichlorophenol	18.5	33	33
3,4,5-Trichlorophenol	21.5	25	0
2,3,4,5-TeCP	30.4	33	33
2,3,4,6-TeCP	27.3	67	68
2,3,5,6-TeCP	27.6	0	0[d]
PCP	50.0	0	8
2,4,6-Tribromophenol	22.3	57	57
2,4,6-Triiodophenol	37.1	50	0[d]
2,4,6-Trimethylphenol	11.3	0	0
2,4,6-Trinitrophenol	7.1	0	0

[a] Activities are the averages from three independent trials. Standard errors are ±5.5% in the HPLC test and ±2.6% in the NADH test. Abbreviations: CP, chlorophenol; DCP, dichlorophenol; TeCP, tetrachlorophenol.

[b] Substrate conversion was determined by HPLC analysis with eluent 1, as described in Materials and Methods.

[c] NADH consumption in the presence of substrate was recorded at 340 nm and corrected for unspecific NADH consumption in the absence of substrate.

[d] Inhibition of unspecific NADH consumption, previously determined in the absence of substrate.

dichlorophenol, and 3,4,5-trichlorophenol may result from the negative influence of these chlorophenols on unspecific NADH consumption which compensates substrate-specific cofactor consumption. In the presence of 2,4,6-triiodophenol, the rate of NADH conversion was even lower than in its absence, although this TCP analog was found to be converted in the HPLC test. On the basis of our experience, the HPLC test is more reliable for the evaluation of substrate conversion than the NADH test, which was found to be more subject to interference and was applied predominantly for routine investigations.

Enzyme localization and organization. Membrane proteins prepared by ultracentrifugation and subsequent solubilization with Triton X-100 showed no TCP-4-monooxygenase activity. Only the cytoplasmic proteins were active in the presence of cofactors, suggesting a cytoplasmic localization and no membrane localization of the enzyme.

The activity of TCP-4-monooxygenase showed a linear dependence on protein concentration, indicating that this enzyme consists of one component and is not organized as a multicomponent enzyme system. No evidence for the involvement of a second protein in the enzyme reaction was obtained.

Molecular mass and kinetic data. The molecular mass of native TCP-4-monooxygenase was determined to be 240 kDa by gel filtration on Superose 12 and on Sephadex G200. This value was confirmed by the sedimentation coefficient $S_{20,W}$ of 10.9 obtained by analytical ultracentrifugation. From SDS-PAGE (Fig. 2), a subunit molecular mass of 60 kDa was calculated, suggesting a homotetrameric structure for TCP-4-monooxygenase. After cross-linking of TCP-4-monooxygenase subunits with dimethylsuberimidate, SDS-PAGE revealed the formation of dimeric (120-kDa), trimeric (180-kDa), and tetrameric (240-kDa) protein species.

Assuming a relative molecular mass of 240 kDa for the enzyme, the specific activity of 6.87 nmol of substrate converted per min and per mg of protein in the purest sample (Table 1) indicates a k_{cat} of 0.029 s^{-1}.

From three independent experiments, the apparent K_m values (means ± standard deviations) for TCP and NADH were estimated as 5 ± 0.4 and 125 ± 8 μM, respectively, and the apparent V_{max} for TCP and NADH was estimated as 25 ± 2 mU/mg. At TCP concentrations higher than 100 μM, substrate inhibition which amounted to 43% at 200 μM TCP and to 72% at 330 μM TCP was observed. Reaction velocity was found to depend also on the FAD concentration, and on the basis of a substrate saturation curve the FAD concentration for half-maximum velocity was determined to be 2.8 ± 0.2 μM (dissociation constant K_d). FAD was observed to be inhibitory at concentrations exceeding 50 μM.

The isoelectric point (pI) was determined as pH 5.2.

Stability and dependence on pH, ionic strength, and temperature. Enzyme activity was completely lost after 2 to 3 days at 4°C and after 8 to 15 h at room temperature. At −20°C, the enzyme was stable for several months. The addition of dithiothreitol (0.5 mM), glycerol (10%, vol/vol), sucrose (10%, wt/vol), or bovine liver catalase (30 ng/ml), separately or in combinations, only slightly enhanced enzyme stability. No influence on stability was found for ethanol (10%, vol/vol), isopropanol (10%, vol/vol), or H_2O_2 (0.1 mM); FAD (0.03 mM) even led to a slight destabilization. Because no effective stabilizing agents were discovered, all efforts were directed at minimizing the time required to purify the protein.

Optimal activity was measured between pH 6.5 and 7.0. An optimum was found with 25 to 50 mM Tris-HCl buffer, pH 7.0. The temperature optimum was observed to be between 35 and 40°C.

TCP-mo (1) M I R T G T Q Y L E S L N D G R N V W V G N E K (24)....

HadA (1) M I R T G T Q Y L E S L N D G R N V W V G N E K....I (518)

P2c-o (1) M R T G K Q Y L K S L N D G R T V I L D G E V....H (30)..

4Hpa-h (12) P F T G E E Y L K S L Q D G R E I Y I Y G E R....K (520)

FIG. 5. N-terminal amino acid sequences exhibiting a high degree of homology from two dehalogenating and two nondehalogenating monooxygenases. Abbreviations: TCP-mo, TCP-4-monooxygenase from *Azotobacter* sp. strain GP1 (this paper); HadA, TCP-4-dechlorinase from *P. pickettii* DTP0602 (24); P2c-o, pyrrole-2-carboxylate oxygenase from *Arthrobacter* sp. strain Py1 (11); 4Hpa-h, 4-hydroxyphenylacetate 3-hydroxylase from *E. coli* (18). Amino acids identical with those from TCP-4-monooxygenase of *Azotobacter* sp. strain GP1 are indicated (boldface).

Influence of metal ions and inhibitors. The addition of 0.1 mM Cu^{2+}, Hg^{2+}, and Zn^{2+} led to inhibitions of 100, 87, and 31%, respectively. No influence on TCP-4-monooxygenase was observed for 1 mM Mg^{2+}, Ca^{2+}, and Co^{2+}; 0.1 mM Fe^{2+}, Fe^{3+}, Co^{2+}, and Ni^{2+} slightly activated the enzyme. X-ray fluorescence analysis provided no evidence for the presence of Ca, Mn, Ni, Co, Cu, Zn, Sr, or Pb in purified TCP-4-monooxygenase. A nonstoichiometric amount of 0.2 mol of Fe per mol of the homotetrameric protein was measured.

Sulfhydryl modifying agents, such as *p*-hydroxymercuribenzoate and *N*-ethylmaleimide (0.1 mM), inhibited the enzyme to 62 and 42%, respectively. The flavin inhibitors quinacrine and acriflavine (0.1 mM) totally inhibited TCP-4-monooxygenase, a result which is in accordance with the FAD requirement of the enzyme. The cytochrome P-450 inhibitors menadione and metapyrone, the metal-chelating agents EDTA, Tiron, and 1,10-phenanthroline, and other inhibitors (KCN and sodium azide) were all found to have no effect at 1 mM.

N-terminal amino acid sequence. The occurrence of a single N terminus for TCP-4-monooxygenase is in accordance with the proposed structure of a protein composed of identical subunits. Figure 5 presents the 24 amino acids of TCP-4-monooxygenase together with homologous protein sequences from another three monooxygenases. The nucleotide-deduced amino acid sequence of the *hadA* locus from the TCP-degrading *P. pickettii* (24) was adopted from the literature. The other two sequences from nondehalogenating aromatic monooxygenases were obtained by broad-range database research for proteins exhibiting more than 40% identity to the N terminus of TCP-4-monooxygenase in the amino acid sequence databases SwissProt and PIR.

H_2O_2 formation by unproductive NADH conversion. The pure TCP-4-monooxygenase exhibited unspecific NADH oxidation leading to the formation of H_2O_2 as demonstrated with the aid of an O_2 electrode after addition of catalase to an enzyme assay (Fig. 6a). TCP-independent NADH consumption occurs by the transfer of hydrogen atoms and electrons from NADH on molecular O_2 to produce H_2O_2. As shown in Fig. 6b, this uncoupling reaction is reduced if TCP is added during the incubation of NADH plus enzyme.

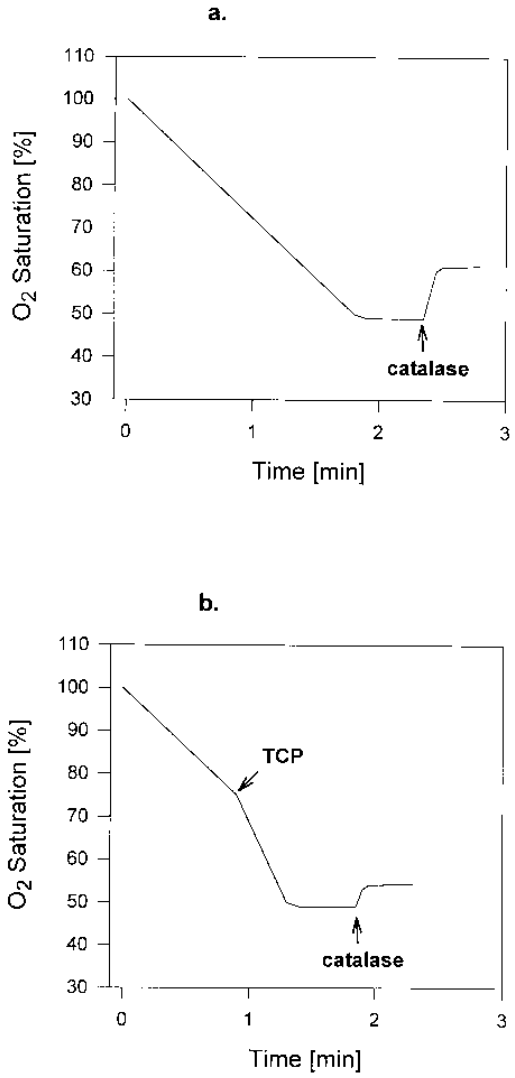

FIG. 6. Production of H_2O_2 during the aerobic reaction of purified TCP-4-monooxygenase from *Azotobacter* sp. strain GP1 (20 μg in 0.8 ml of buffer A containing 0.03 mM FAD) with 0.25 mM NADH, in the absence of TCP (a) and with TCP addition to a final concentration of 0.1 mM (b). Catalase (1 μg from bovine liver) was added at the indicated times (arrows).

DISCUSSION

In this paper, we describe the purification and properties of the enzyme which catalyzes the initial step of TCP degradation in *Azotobacter* sp. strain GP1, a strain which was previously shown to be very efficient in the degradation of this specific trichlorophenol (14). For the conversion of TCP to 2,6-dichlorohydroquinone, O_2, NADH, and FAD are required, and according to the rules for recommended names assigned by the Nomenclature Committee of the International Union of Biochemistry (30), the enzyme should therefore be named TCP-4-monooxygenase.

TCP-4-monooxygenase is induced during growth on TCP. The inability of phenol, which is another growth substrate for *Azotobacter* sp. strain GP1, to induce the TCP-dehalogenating enzyme suggests that this organism degrades phenol and TCP via different pathways. This agrees with findings obtained by induction experiments with whole cells in the presence and absence of chloramphenicol (14) and with results concerning the metabolism of phenol (32).

TCP induction led to relatively large amounts of the dehalogenating enzyme, as shown by SDS-PAGE. Data from Table 1 suggests that the enzyme represents about 3% of the soluble protein, a value which is significantly higher than for other polychlorophenol-dehalogenating enzymes, such as pentachlorophenol-4-monooxygenase (PCP-4-monooxygenase) from a *Flavobacterium* sp. (0.1%) (34) and chlorophenol-4-monooxygenase from *B. cepacia* (0.02% for component A [cA] and

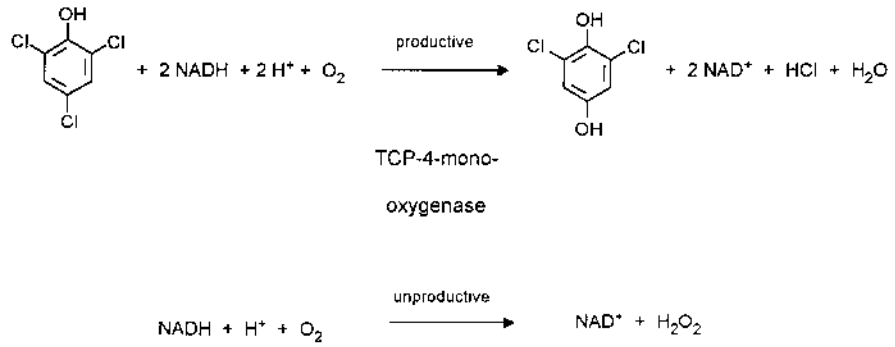

FIG. 7. Reactions catalyzed by TCP-4-monooxygenase from *Azotobacter* sp. strain GP1.

0.006% for component B [cB]) (33). This strong enhancement of enzyme production may partly compensate the low activity indicated by the catalytic constant of 0.027 s^{-1} for the pure enzyme and by a specific activity of 0.18 mU/mg for the crude extract. For comparison, PCP-4-monooxygenase from the *Flavobacterium* sp. showed a specific activity of 11,540 mU/mg in the crude extract and a k_{cat} of 12.1 s^{-1}. The dehalogenase from *B. cepacia* exhibited 11 mU/mg for cA and 7 mU/mg for cB and a k_{cat} of 0.306 s^{-1}. Crude extract activities even smaller than for the *Azotobacter* enzyme were determined for the PCP-dehalogenating enzymes from *M. fortuitum* (28) and *R. chlorophenolicus* (27) (0.03 mU/mg for both enzymes in the crude extract). This wide range of activities found for functionally similar enzymes is probably due to differences in enzyme stability during cell disruption of the different organisms. According to this assumption, the greatest part of activity is lost upon breakage of cells of *M. fortuitum*, *R. chlorophenolicus*, and the organism of this study. With whole cells of the *Azotobacter* sp., a maximum degradation rate for TCP of 42 mU/mg was determined (14). This in vivo rate is significantly higher than the dehalogenating activity in vitro (0.18 mU/mg), supporting the hypothesis that activity might be lost during preparation of the crude extract. Also, other enzymes from *Azotobacter* sp. strain GP1 acting in the degradative pathway of TCP (hydroxyquinol 1,2-dioxygenase and maleylacetate reductase) were found to exhibit a significantly higher activity than TCP-4-monooxygenase (13, 36).

No evidence for membrane association, as observed for the PCP-dehalogenating enzyme from *R. chlorophenolicus* PCP-1 (26, 27) and from *M. fortuitum* CG-2 (28), was obtained for TCP-4-monooxygenase. Enzyme activity was shown to be located in the cytoplasm, as for the PCP-4-monooxygenases from the *Flavobacterium* sp. (34) and *B. cepacia* (33). The enzyme from the *Flavobacterium* sp. was also shown to be organized as a one-component enzyme, like TCP-4-monooxygenase from *Azotobacter* sp. strain GP1. The enzyme from *B. cepacia*, however, was found to consist of two components, cA and cB. Neither of the components alone dehalogenated 2,4,5-trichlorophenol or TCP; only the combination catalyzed this reaction. For the analogous enzyme from *P. pickettii*, genetic evidence for two open reading frames, *hadA* and *hadB*, suggested its organization as a two-component enzyme, although the product of the *hadB* gene was not essential for the expression of dechlorinating activity in vitro (24). The involvement of a second protein in the dehalogenating reaction of the *Azotobacter* sp. provides another possibility to explain the low catalytic activity of the enzyme. However, attempts to detect a second protein in the TCP-4-monooxygenase reaction in *Azotobacter* sp. strain GP1 were without success.

The purified TCP-4-monooxygenase, although shown to be FAD dependent, did not exhibit a typical flavoprotein spectrum. The relatively high K_d (FAD) of 2.8 μM for the *Azotobacter* enzyme compared to values from 0.039 to 0.28 μM for other monooxygenases (1, 3, 20) suggests that FAD is a loosely bound cofactor. The total amount of FAD is lost during enzyme purification, and the loss is probably enhanced by the presence of high salt concentrations. The purified protein represents the apoenzyme, which has to be supplemented by FAD addition to obtain full activity.

The 24 N-terminal amino acid residues of TCP-4-monooxygenase from *Azotobacter* sp. strain GP1 were found to be identical with the N terminus of an analogous enzyme component (HadA) from the TCP-degrading *P. pickettii* strain DTP0602 (24). Sequence data for the *Azotobacter* enzyme were obtained by sequencing of the purified protein, whereas the data for the *Pseudomonas* enzyme are deduced from the nucleotide sequence of the *hadA* locus. For in vitro expression of the dechlorinase, only the HadA product with the same N terminus as the *Azotobacter* enzyme, not a second component named HadB, was necessary. N-terminal sequence identity is an impressive coincidence of results, obtained by two independent methods, and it seems worthwhile to complete this sequence comparison.

Sequence alignment of the hadA gene product from *P. pickettii* with PCP-4-monooxygenase from the *Flavobacterium* sp. (16, 17), using the HUSAR program CLUSTAL from the University of Heidelberg, revealed a relatively low homology (14.5% identical amino acid residues). In accordance with this result, the N terminus of the *Azotobacter* enzyme shows only 17% identity with the N-terminal sequence of PCP-4-monooxygenase from the *Flavobacterium* sp. The N termini of two nondehalogenating monooxygenases, pyrrole-2-carboxylate oxygenase from *Arthrobacter* sp. strain Py1 (11) and 4-hydroxyphenylacetate 3-hydroxylase from *Escherichia coli* (18), showed high degrees of homology (58 and 42% identity, respectively) with the *Azotobacter* enzyme (Fig. 5). This is an indication for the relatedness of dehalogenating and nondehalogenating monooxygenases. This group of flavoproteins which all depend on the same cosubstrates, namely, flavin, O_2, and NAD(P)H, have also the subunit size of about 60 kDa in common (3, 11, 19, 29, 34). They catalyze the hydroxylation of an aromatic substrate either *ortho* or *para* relative to the existing hydroxyl group. TCP-4-monooxygenase is able to hydroxylate the aromatic nucleus in *para* position also in the absence of a chlorine substituent: 2,6-dichlorophenol is converted to 2,6-dichlorohydroquinone in a nondehalogenating reaction. Similarly, PCP-4-monooxygenase from a *Flavobacterium* sp. (35) catalyzes the hydroxylation of 2,3,5,6-tetrachlorophenol to tetrachlorohydro-

quinone, and this nondehalogenating reaction is much faster than the dehalogenation of PCP. These results emphasize a close relationship of dehalogenating and nondehalogenating monooxygenases.

As stated by Neujahr (15), unproductive NAD(P)H oxidation is observed with all aromatic flavoprotein monooxygenases. This uncoupling of oxygen reduction from hydroxylation, which was first intensively studied for salicylate-hydroxylase from *Pseudomonas putida* (31), occurs in most cases in the presence of a pseudosubstrate, and H_2O_2 is the reaction product. However, also in the absence of a false substrate, NADPH oxidation together with H_2O_2 and H_2O production was found (8). For *p*-hydroxyphenylacetate-3-hydroxylase from *P. putida*, a second, colorless protein, referred to as the coupling protein, was required for product formation (2). The flavoprotein alone in the presence of substrate and substrate analogs catalyzes the wasteful oxidation of NADH with the stoichiometric generation of H_2O_2. Similar observations were reported for 4-hydroxybenzoate 3-hydroxylase from *E. coli* (18), where the flavoprotein alone was able to catalyze product formation, although at a considerably reduced rate.

In this paper, unproductive NADH oxidation was described also for a dehalogenating monooxygenase (Fig. 7). This finding adds a further property common to dehalogenating and nondehalogenating monooxygenases.

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