Purification and Characterization of Hydroxyquinol 1,2-Dioxygenase from *Azotobacter* sp. Strain GP1

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Hydroxyquinol 1,2-dioxygenase was purified from cells of the soil bacterium *Azotobacter* sp. strain GP1 grown with 2,4,6-trichlorophenol as the sole source of carbon. The presumable function of this dioxygenase enzyme in the degradative pathway of 2,4,6-trichlorophenol is discussed. The enzyme was highly specific for 6-chlorohydroxyquinol (6-chloro-1,2,4-trihydroxybenzene) and hydroxyquinol (1,2,4-trihydroxybenzene) and was found to perform *ortho* cleavage of the hydroxyquinol compounds, yielding chloromaleylacetate and maleylacetate, respectively. With the conversion of 1 mol of 6-chlorohydroxyquinol, the consumption of 1 mol of O₂ and the formation of 1 mol of chloromaleylacetate were observed. Catechol was not accepted as a substrate. The enzyme has to be induced, and no activity was found in cells grown on succinate. The molecular weight of native hydroxyquinol 1,2-dioxygenase was estimated to 58,000, with a sedimentation coefficient of 4.32. The subunit molecular weight of 34,250 indicates a dimeric structure of the dioxygenase enzyme. The addition of Fe²⁺ ions significantly activated enzyme activity, and metal-chelating agents inhibited it. Electron paramagnetic resonance data are consistent with high-spin iron(III) in a rhombic environment. The NH₂-terminal amino acid sequence was determined for up to 40 amino acid residues and compared with sequences from literature data for other catechol and chlorocatechol dioxygenases.

Chlorophenols are widely used as disinfectants and preservatives and have entered the environment in considerable amounts (18, 25).

Whereas the microbial degradation of pentachlorophenol and 2,4-dichlorophenol was investigated by numerous groups (see references 14, 15, 28, and 34), only a few reports have dealt with the degradation of 2,4,6-trichlorophenol (TCP) (13, 22, 26). In a previous paper, we have described the isolation and several properties of the TCP-degrading *Azotobacter* sp. strain GP1 (26). 2,6-Dichlorohydroquinone was found as the first intermediate of TCP degradation (see Discussion and Fig. 3). The same intermediate was obtained in the TCP-degrading strains *Pseudomonas pickettii* (22) and *Streptomyces rochei* 303 (13). As the next step in the degradation pathway, we postulated a dechlorination of 2,6-dichlorohydroquinone to 6-chlorohydroxyquinol (CHQ). This compound shows catechol structure and was found to be the substrate of a ring-cleaving enzyme present in *Azotobacter* sp. strain GP1.

In this paper, we describe the purification and characterization of hydroxyquinol 1,2-dioxygenase, an enzyme postulated to be involved in the degradation of TCP (hydroxyquinol [HQ] is 1,2,4-trihydroxybenzene). Another HQ-cleaving enzyme, also assumed to play a role in TCP degradation, was purified and characterized from TCP-induced cells of *S. rochei* 303 (48). In fungi, two other HQ 1,2-dioxygenases have already been purified and characterized. The first enzyme was isolated from the soil yeast *Trichosporon cutaneum* grown with 4-hydroxybenzoate (40); the second one was purified from vanillate-induced cells of the basidiomycete *Phanerochaete chrysosporium* (35).

MATERIALS AND METHODS

Chemicals. TCP was from Merck, Darmstadt, Germany; HQ was from Lancaster Synthesis, Morecombe, England.

6-Chlorohydroxyquinol was obtained in a three-step synthesis. In the first step, vanillin was chlorinated with sodium hypochlorite to 5-chlorovanillin (17). Ether cleavage of 5-chlorovanillin to 3-chloro-4,5-dihydroxybenzaldehyde was performed with aluminum chloride in pyridine (2). CHQ was synthesized by a modified Dakin oxidation (9): 50 mg of 3-chloro-4,5-dihydroxybenzaldehyde was dissolved in 300 μ l of sodium hydroxide (1 N), yielding a dark green solution. When the aldehyde was completely dissolved, 200 μ l of H₂O₂ (30%) was added. A strong exothermic reaction followed. The color turned from green to dark red-brown. The reaction was allowed to continue for 2 min. The solution was acidified by the addition of 2 ml of H₂SO₄ (1 N) and kept on ice. Upon cooling, unreacted aldehyde precipitated. The solution was filtered and extracted twice with ethylacetate. The extracts were combined, washed with acidified saturated sodium chloride solution (5 ml), and dried over anhydrous sodium sulfate. After decanting from sodium sulfate, ethylacetate was concentrated to dryness in a rotavapor, and the residue was dried in a desiccator over calcium chloride in an argon atmosphere. To avoid auto-oxidation and polymerization, synthesis and storage were performed under an argon atmosphere and in the dark. The synthesized CHQ showed a mass spectrum consistent with the proposed structure, and it was found to be identical in UV spectrum to a sample kindly provided by L. A. Golovleva, Academy of Sciences, Pushchino, Moscow Region, Russia.

Maleylacetate and chloromaleylacetate were synthesized by alkaline hydrolysis with 4 N NaOH at 0°C of butenolide (4-carboxymethylene but-2-enolide) and chlorobutenolide (2-chloro-4-carboxymethylene but-2-enolide), respectively. The production of maleylacetate was monitored spectrophotometrically at 245 nm; that of chloromaleylacetate was monitored at 253 nm. Butenolide and chlorobutenolide were synthesized by S. Kaschabek with the aid of the Wittig reaction between stabilized phosphoranes and maleic anhydride or chloromaleic anhydride (21).

Organism and growth conditions. The isolation and characterization of *Azotobacter* sp. strain GP1 have been described previously (26). *Azotobacter* sp. strain GP1 is deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany, under the accession number DSM 6428 and at the American Type Culture Collection, Rockville, Md., under the accession number ATCC 49806. The bacterium was grown in a 20-liter fermentor inoculated with a 50-ml preculture in mineral salt medium containing the following (in grams per liter): K₂HPO₄, 1.73; KH₂PO₄, 0.68; NH₄NO₃, 1.0; MgSO₄ · 7H₂O, 0.1. The solution, containing these components, was autoclaved separately. The filter-sterilized salts (final concentrations in grams per liter), namely, MnSO₄ · H₂O (0.03), FeSO₄ · 7H₂O (0.03), and CaCl₂ · 2H₂O (0.02), and succinate as a carbon source at a concentration of 4 g/liter were added aseptically. At the end of the logarithmic growth phase, cells were induced by the addition of TCP (200 mg/liter). After degradation of approximately 80% of the TCP, cells were har-

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vested by centrifugation and washed twice with phosphate buffer (20 mM). The cells were stored at -20°C for several weeks without loss of activity.

Enzyme assays. (i) HQ 1,2-dioxygenase. (a) UV test. During enzymatic conversion of CHQ, a distinctive peak appeared at 253 nm, which vanished when the solution was acidified to pH 2. This is a characteristic feature of chloromaleylacetate. Similar spectroscopic characteristics were found during conversion of HQ with a distinctive peak at 245 nm, which indicated the formation of maleylacetate (molar extinctions for both compounds were reported by Tiedje et al. [41]). Tests were performed at 25°C in 1-ml cuvettes containing phosphate buffer (10 mM, pH 7.0) and protein. The reaction was started by the addition of substrate. One unit of activity is the amount of HQ 1,2-dioxygenase that catalyzes the formation of 1 μmol of chloromaleylacetate per min. For each test, the substrates were freshly prepared as 10 mM solutions in bidistilled water under an argon atmosphere. Nonenzymatic conversion of substrate was subtracted from substrate conversion in the presence of enzyme.

(b) O_2 test. O_2 consumption was measured with a Clark DW 1 O_2 electrode from Bachofer, Reutlingen, Germany. To the 1-ml reaction mixture, containing phosphate buffer (10 mM, pH 7.0) or water, substrate was added to a final concentration of 0.2 mM and preincubated at 25°C for 1 min to determine endogenous O_2 consumption. The reaction was started by the injection of protein. One unit of activity is the amount of HQ 1,2-dioxygenase that catalyzes the uptake of 1 μmol of O_2 per min.

(ii) Maleylacetate reductase. The maleylacetate reductase test was performed as described by Kaschabek and Reineke (20). A total volume of 1 ml contained 3 to 30 μg of protein (crude extract) and 0.25 or 0.1 mM of the substrates (NADH and either maleylacetate or chloromaleylacetate) in 10 mM phosphate buffer (pH 7.0). NADH consumption was also observed in crude extract without the addition of substrate and was subtracted from the enzyme reaction.

Inhibition and activation studies. Because of the low yield of pure enzyme, in inhibition experiments, partially purified HQ 1,2-dioxygenase from Mono Q column chromatography (75 μg per assay) was applied. The influence of iron ions was tested with the pure enzyme from Superdex 75 column chromatography (12 μg per assay). After different times of enzyme incubation (up to 6 h), the enzyme reaction was started by the addition of 0.2 mM HQ as the substrate. Before testing the activity, excessive iron was removed by ultrafiltration with repeated additions of iron-free buffer. Activity was determined with the O_2 test.

Detection of chloromaleylacetate. Synthesized chloromaleylacetate and the product of enzymatic conversion of CHQ by HQ 1,2-dioxygenase were subjected to high-performance liquid chromatography (HPLC) with a Gromsil column (125 by 4.6 mm) filled with 5- μm particles of LiChrospher 100 RP8 from Bischoff, Leonberg, Germany. An aqueous solution of 40% (vol/vol) methanol and 0.1% (vol/vol) H_3PO_4 was used as the eluent. The compounds were detected at 210 nm. Samples from the enzyme-catalyzed reaction were acidified with an appropriate volume of the eluent and centrifuged before injection.

Analytical methods. UV spectra were recorded with a Kontron Uvicon 930 or a Zeiss-DMR 21 spectrophotometer.

The protein concentration was determined by the method of Bradford (7) with bovine serum albumin as the standard.

Molecular weight was estimated by fast protein liquid chromatography (FPLC) gel filtration with a Superdex 75 column (16/6, preparation [prep] grade). The eluent was phosphate buffer (50 mM) containing 0.15 M KCl with a flow rate of 1 ml/min. The M_r was calculated from a standard linear regression curve of reference proteins (M_r of 25,000 to 78,000).

The sedimentation coefficient was estimated by ultracentrifugation in a model E analytical centrifuge from Beckman Instruments, Munich, Germany, at 60,000 rpm with 0.5 ml of purified enzyme solution in phosphate buffer (50 mM) containing 0.15 M KCl with an A_{280} of 0.6.

Enzyme purity and molecular weight of the subunits were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Schägger and von Jagow (36) in 10 to 12.5% gradient gels with low-molecular-weight standard proteins (M_r s of 14,400 to 94,000) from Pharmacia, Uppsala, Sweden. The proteins were stained with Coomassie blue R250. The M_r was calculated from a standard linear regression curve of reference proteins.

Isoelectric focusing with a polyacrylamide gel (4.2%) and a pH gradient from 4 to 6 with reference proteins (pI 4.15 to 5.85) allowed the determination of the isoelectric point of HQ 1,2-dioxygenase with the aid of a standard curve.

The metal content of HQ 1,2-dioxygenase was determined by X-ray fluorescence spectroscopy (system 77; Finnigan International, Inc., Sunny Valley, Calif.

Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ER 420 spectrometer equipped with a continuous-helium-flow cryostat (model ESR 900). The magnetic field and the microwave frequency were determined with a nuclear magnetic resonance gaussmeter and a microwave counter, respectively. Samples of purified protein (7 mg; approximately 30 U) in a total volume of 200 μl were transferred into quartz tubes and frozen in liquid nitrogen within 1 min.

Determination of acid-labile sulfur was done as described by Beinert (5).

The NH_2 -terminal amino acid sequence was determined by applying automatic Edman degradation with a model 471 A protein sequencer from Applied Biosystems, Weiterstadt, Germany.

Purification of HQ 1,2-dioxygenase. All purification steps, with the exception of FPLC applications, were carried out at 4°C . The frozen cells (60 g [wet

TABLE 1. Specific activities of ring-cleaving enzymes in crude extracts from uninduced, phenol-induced, and TCP-induced cells, tested with different substrates^a

Crude extract	Sp act (mU/mg) with:		
	Catechol ^b	HQ ^b	CHQ ^b
Uninduced	0	6.1	3.0
Phenol induced	120.6 ^c	137.0 ^c	10.2
TCP induced	5.1	546.4	341.8

^a Uninduced cells were grown on succinate. Phenol-induced cells were grown as described for TCP-induced cells in Materials and Methods, with 200 mg of phenol per liter used for induction. Harvesting and cell disruption were as described in Materials and Methods. Activity was determined with the O_2 test with 1 mg of crude extract and 0.2 mM substrate.

^b Substrate used in dioxygenase test.

^c Conversion of substrate by *meta* cleavage, as indicated by the absorption spectra of the products.

weight)] were suspended in 60 ml of phosphate buffer (100 mM, pH 7.0) and disrupted by a 20-min sonication with a Branson model 450 ultrasonic disintegrator. Cell debris and intact cells were removed by centrifugation at $40,000 \times g$ for 40 min. The supernatant (80 ml) was designated as crude extract.

To the crude extract, powdered ammonium sulfate was added slowly to 25% saturation. The mixture was stirred gently for 45 min, and the precipitate was removed by centrifugation at $40,000 \times g$ for 40 min and discarded. The supernatant was dialyzed against 5 liters of phosphate buffer (20 mM, pH 7.0) overnight for the removal of ammonium sulfate.

The dialyzed supernatant (~100 ml) was loaded on a Q-Sepharose Fast Flow ion-exchange column previously equilibrated with phosphate buffer (20 mM, pH 7.0) and washed with 400 ml of the same buffer. The enzyme was eluted with 500 ml of a linear concentration gradient of 0 to 0.4 M KCl in buffer. The enzyme was found to elute at 0.25 M KCl. Active fractions were pooled and concentrated by ultrafiltration (Amicon ultrafiltration cell with a PM-10 membrane).

The concentrated pool from the Q-Sepharose column was applied to a calcium tartrate column synthesized by the method of Akhrem and Drozhdenyuk (1). From the column previously equilibrated with phosphate buffer (10 mM, pH 7.0), the enzyme was washed with the same buffer. Active fractions were pooled and concentrated as described for the previous step.

The concentrated supernatant from calcium tartrate chromatography was applied to a Mono Q column (0.5 by 5 cm) in the Pharmacia FPLC system in repeated runs. The gradient and buffers were identical to those used in chromatography with the Q-Sepharose column. Active fractions were pooled and concentrated to 0.5 ml in a Centricon 10 microconcentrator.

The combined and concentrated pools from the Mono Q step were loaded on a Superose 12 gel filtration column in the Pharmacia FPLC system, previously equilibrated with phosphate buffer (50 mM, pH 7.0) containing 0.15 M KCl, and eluted at a flow rate of 30 ml/h. Before application, the supernatant was centrifuged to remove particles and insoluble protein ($40,000 \times g$, 20 min). Active fractions were pooled and concentrated to a final volume of 0.5 ml in a Centricon 10.

Finally, a Superdex 75 prep-grade column (16/6, prep grade) in a Pharmacia FPLC system was loaded with the concentrated protein from the Superose 12 pool. The column was equilibrated with phosphate buffer (50 mM, pH 7.0) containing 0.15 M KCl. The flow rate for equilibration and subsequent elution was 60 ml/h. Active fractions were combined and concentrated to 0.5 ml in a Centricon 10. The purified enzyme was stored at -80°C .

RESULTS

Induction of HQ 1,2-dioxygenase. The results of Table 1 present evidence for the inducible nature of HQ 1,2-dioxygenase. Cells grown on succinate did not show enzyme activity. Only with a TCP-induced extract was *ortho* cleavage of HQ and CHQ, suggesting the involvement of this enzyme in the degradation of the trichlorinated phenol, observed. For the dissimilation of nonchlorinated phenol, the existence of a second independent pathway in *Azotobacter* sp. strain GP1 has been shown previously with the aid of catabolic mutants (26). As demonstrated by Wieser et al. (44), the degradation of nonchlorinated or monochlorinated phenol proceeds via the *meta* cleavage pathway. Incubation of HQ with the phenol-induced extract led to significant O_2 consumption; however, the obser-

TABLE 2. Purification of HQ 1,2-dioxygenase^a

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
Crude extract	4,026	431	0.11	100
Ammonium sulfate fractionation	2,900	319	0.11	74
Q-Sepharose Fast Flow	758	318	0.42	74
Calcium tartrate	349	305	0.87	71
Mono Q	153	148	0.97	34
Superose 12	70	68	0.97	16
Superdex 75 ^b	5.8	40.1	7.06	10.8

^a Activity was measured with an O₂ electrode with 0.2 mM HQ as the substrate as described in Materials and Methods.

^b Reactivated with 0.01 mM Fe²⁺.

vation of a yellow product suggested *meta* instead of *ortho* cleavage of the 1,2,4-trihydroxybenzene. This assumption was confirmed by the observation of the typical absorption maximum of the *meta* cleavage product (2-hydroxymuconate semialdehyde) at 375 nm when catechol as the substrate was tested with extracts from phenol-induced cells. In the same extracts, the values of O₂ consumption, tested with CHQ as the substrate, were comparable to the values of O₂ consumption in extracts of uninduced cells, suggesting unspecific oxidation of the HQ compounds by the crude extracts.

Purification of HQ 1,2-dioxygenase. Table 2 summarizes the purification of the enzyme. After six steps, 5.8 mg of a protein, homogeneous by SDS-PAGE (data not shown), was obtained with 66-fold enrichment. If not otherwise stated, all investigations were performed with a pure enzyme preparation.

Reaction catalyzed by HQ 1,2-dioxygenase. During incubation of CHQ with HQ 1,2-dioxygenase, the substrate peak at 278 nm disappeared, and as described by Zaborina et al. (48), a new peak was observed at 253 nm. This peak vanished when the enzyme reaction mixture was acidified. The spectral behavior of the reaction product is typical for chloromaleylacetate. Similar results were obtained with HQ: the substrate peak at 278 nm disappeared, giving rise to a new peak at 245 nm, which again vanished upon acidification, indicating the presence of maleylacetate in the reaction mixture. The identity of chloromaleylacetate was further confirmed by a comparison of the enzyme reaction product with a synthetic sample by HPLC. Chloromaleylacetate obtained from chlorobutenolide hydrolysis showed a retention volume of 1.72 ml, and in a separate run, the ring cleavage product with CHQ as the substrate exhibited an almost identical retention volume of 1.73 ml. Table 3 presents the results on the stoichiometry of the reaction catalyzed by HQ 1,2-dioxygenase: the molar ratio of CHQ consumed/chloromaleylacetate formed/O₂ consumed approximates 1:1:1.

TABLE 3. Stoichiometry of the reaction catalyzed by HQ 1,2-dioxygenase^a

CHQ supplied (μmol)	Chloromaleylacetate formed (μmol)	O ₂ consumed (μmol)
100	104.3	91.1
200	192	187
400	404	375

^a After the addition of an excessive amount of HQ 1,2-dioxygenase (15, 30, or 60 μg/ml), which guaranteed a complete conversion of CHQ after 6 min, formation of chloromaleylacetate was determined spectrophotometrically (A₂₅₃), and the consumption of oxygen was determined with the O₂ electrode as described in Materials and Methods.

TABLE 4. Influence of Fe²⁺ and chelating agents on activity of HQ 1,2-dioxygenase

Effector ^a	Relative activity (%) ^b at effector concn of:			
	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴	10 ⁻³
DDTC		100	100	100
EDAS		97	81	77
EDTA		80	86	78
Tiron		56	65	17
Fe ²⁺	211	555	ND ^c	ND

^a Abbreviations: DDTC, diethyldithiocarbamate; EDAS, ethylenediammoniumsulfate; Tiron, catechol-3,5-disulfonate; Fe²⁺, ammonium iron(II) sulfate hexahydrate.

^b Activity after 1 h of incubation in the presence of effector was determined as described in Materials and Methods. Activity without effector was 100%.

^c ND, not determined because of increased auto-oxidation of HQ in the presence of high Fe²⁺ concentrations.

Substrate specificity. HQ 1,2-dioxygenase was found to be highly specific for 1,2,4-trihydroxybenzenes. From 17 polyhydroxybenzenes tested, only HQ and CHQ were accepted as substrates. The relative activity with CHQ as the substrate was 33.5% of that measured with HQ (100%). None of the three hydroquinones (hydroquinone, 2,6-dichlorohydroquinone, and 2,3,6-trichlorohydroquinone), the eight catechols (catechol, 3-methylcatechol, 3- and 4-chlorocatechol, 3,5-dichlorocatechol, 5-chloro-3-methylcatechol, 3,4-dihydroxybenzoic acid, and 3,4-dihydroxyphenylacetic acid), or the two pyrogallols (pyrogallol and 3,4,5-trihydroxybenzoic acid) was oxidized by the enzyme. Also, two compounds exhibiting an HQ structure, namely, 3,5,6-trichlorohydroxyquinol and 2,3,5-trihydroxybenzoic acid, did not serve as substrates. There was no observation of extradiol ring cleavage.

Inhibition and activation studies. Table 4 shows the influence of metal-chelating agents and of Fe ions on the activity of HQ 1,2-dioxygenase. The enzyme lost more than 80% of its activity when preincubated with 10⁻³ M Tiron (i.e., catechol-3,5-disulfonate) for 1 h; with 10⁻² M and incubation for 1 h, the enzyme was completely inhibited. Preincubation with ethylenediammonium sulfate and EDTA led to only a slight decrease in activity. HQ 1,2-dioxygenase was activated significantly by incubation with 10⁻⁵ and 10⁻⁶ M ammonium iron(II) sulfate. With 10⁻⁷ M Fe²⁺, no increase in activity was observed. Activation was found to be time dependent and reached a final value after 1 h of incubation.

Stability and kinetic properties of HQ 1,2-dioxygenase. The enzyme was reasonably stable under different conditions. If stored at -80°C, no loss of activity was found after several weeks. At room temperature, no considerable decrease occurred after 1 day. At 40°C and within 30 min, no activity was lost; at 50, 60, and 70°C, half-lives of 15, 8, and 1 min, respectively, were observed. The highest activity was found after 5 min of preincubation at 30°C.

Optimum activity in succinate buffer (10 mM) was measured at pH 6.4. Since CHQ undergoes rapid auto-oxidation under basic conditions, it was not possible to determine the pH dependence of the enzyme reaction for values higher than 7.4.

Kinetic data for HQ 1,2-dioxygenase from *Azotobacter* sp. strain GP1 were published by Zaborina et al. (48). Values for *K_m*, *V_{max}*, and *k_{cat}* with HQ as the substrate were very similar to those obtained for the analogous enzyme from *S. rochei* 303. The substrate CHQ, however, was accepted much better by the *Streptomyces* enzyme.

Physical and chemical properties of HQ 1,2-dioxygenase. At high concentrations, the enzyme showed a light red to brown

FIG. 1. Absorption spectrum of purified HQ 1,2-dioxygenase in 10 mM Tris-HCl, measured against a blank of the same buffer. A total volume of 0.2 ml contained an enzyme solution of 8.4 mg of protein per ml with 2.63 U/mg.

color, and in a light-absorption spectrum, a peak at 279 nm and a faint shoulder around 420 nm were measured (Fig. 1). By X-ray fluorescence spectroscopy, no evidence for the presence of calcium, manganese, nickel, lead, and copper was obtained; however, 0.9 mol of zinc and 0.18 mol of iron per mol of enzyme were detected. The enzyme was found to contain no acid-labile sulfur.

The EPR spectrum of the purified enzyme at 5 K is shown in Fig. 2. Resonances are centered at g -tensors of 4.3 (majority component) and 9.67 (minority component), which is characteristic for mononuclear high-spin Fe(III) in a rhombic environment. No characteristic signals for iron-sulfur components were found. Spectra at different temperatures (5, 30, and 73 K) did not provide evidence for the presence of other metallic cofactors.

The molecular weight of native HQ 1,2-dioxygenase was estimated to be 58,000 by gel filtration with a Superdex 75 column and 58,500 by sedimentation equilibrium centrifuga-

tion. The sedimentation coefficient was calculated to be 4.32. Electrophoresis in an SDS-PAGE system yielded a single band corresponding to a molecular weight of 34,250. It is therefore suggested that the enzyme is a dimer of two identical subunits.

The isoelectric point (pI) was estimated to be 4.5.

The NH_2 -terminal amino acid sequence of HQ 1,2-dioxygenase from *Azotobacter* sp. strain GP1 was determined as follows: Arg Leu Asn Leu Asp Glu Asp Thr Ile Thr Gln Ala Val Leu Ala Arg Leu Ala Asp Thr Pro Asp Ile Arg Val Asp Glu Ile Ile Ala Ala Leu Arg Val Asp Leu Leu Trp Phe His.

Detection of maleylacetate reductase in *Azotobacter* sp. strain GP1. Crude extracts were found to catalyze the conversion of maleylacetate and of chloromaleylacetate when NADH was added. Specific activities with maleylacetate or chloromaleylacetate as the substrate were 477 and 476 mU/mg, respectively.

FIG. 2. EPR spectrum of purified HQ 1,2-dioxygenase. The spectrum was recorded at 5 K with 7 mg of enzyme in 0.2 ml of Tris-HCl buffer. Scan time was 3 min; microwave frequency was 9.58 GHz. An average of three scans is shown. Upper line, amplified spectrum.

FIG. 3. Pathway proposed for the degradation of TCP in *Azotobacter* sp. strain GP1 (for details, see Discussion).

DISCUSSION

Role of HQ 1,2-dioxygenase in TCP degradation. The complete pathway for the dissimilation of polychlorinated phenols, including all of the metabolites and enzymes, has not been described to date. As a first step, a hydroxylation in the para position yielding a chlorohydroquinone was observed for different polychlorinated phenols in various bacteria and fungi (3, 13, 22, 24, 26). In the degradation of TCP by *Azotobacter* sp. strain GP1, this step, which in Fig. 3 was designated dechlorination step 1, leads to 2,6-dichlorohydroquinone. Aspects of the mechanism of this reaction and the properties of the enzyme catalyzing hydroquinone formation have been studied in an *Arthrobacter* sp., a *Flavobacterium* sp., and *Mycobacterium fortuitum* (37, 42, 45, 46).

Only a few reports, however, deal with the following degradative steps. In the case of pentachlorophenol degradation in *Rhodococcus chlorophenolicus*, evidence for a further hydroxylation leading to trichlorohydroxyquinol was provided by gas chromatography-mass spectrometry analysis (4, 16). The assumption of the analogous mechanism in *Azotobacter* sp. strain GP1, namely, the conversion of 2,6-dichlorohydroquinone by the dechlorination step 2 (Fig. 3), led us to postulate CHQ as a subsequent metabolite of TCP degradation. This metabolite may serve as a substrate for *ortho* cleavage, giving rise to chloromaleylacetate formation by the ring cleavage step of Fig. 3. The demonstration of enzymes catalyzing the ring cleavage of CHQ to chloromaleylacetate and the conversion of chloromaleylacetate in *Azotobacter* sp. strain GP1, as described in this

TABLE 5. Comparison of all HQ-cleaving enzymes described to date and two representatives from the catechol-cleaving enzymes by substrate specificity and molecular mass of native enzyme and subunits

Enzyme	Reference or source	Substrate specificity (relative activity [%])	Mol mass of native enzyme (Da)	Mol mass(es) of subunit(s) (Da)
HQ 1,2-dioxygenase from <i>Azotobacter</i> sp. strain GP1	This paper	HQ (100), CHQ (33), catechol (0)	58,500	34,250
CHQ 1,2-dioxygenase from <i>S. rochei</i> 303	48	CHQ (100), HQ (15), catechol (0)	61,000	31,000
HQ 1,2-dioxygenase from <i>T. cutaneum</i>	40	HQ (100), catechol (1)	93,000	38,200, 39,600
HQ 1,2-dioxygenase from <i>Phanerochaete chrysosporium</i>	35	HQ (100), catechol (20)	90,000	45,000
Catechol 1,2-dioxygenase from <i>Pseudomonas putida</i> mt-2 ^a	30	Catechol (100), 3-methylcatechol (9.8), 4-methylcatechol (88.0)	64,000–66,000	32,000
Catechol 1,2-dioxygenase from <i>Pseudomonas putida</i> ^b	8	Catechol (100), 3-methylcatechol (354), 4-methylcatechol (273), 3-chlorocatechol (131), 3,5-dichlorocatechol (83)	57,505	32,000

^a As type I representative.^b As type II representative.

report, suggest the operation of the pathway proposed above in this organism. In the case of TCP degradation in *S. rochei* 303, the involvement of an HQ-cleaving dioxygenase in chlorophenol degradation was proposed for the first time (13). During growth of *S. rochei* 303 on TCP or on 2,6-dichlorophenol, a dioxygenase which is highly specific for CHQ as the ring cleavage substrate was induced. Nonchlorinated HQ was accepted at a much lower rate, suggesting a degradation pathway via 2,6-dichlorohydroquinone, CHQ, and chloromaleylacetate (Fig. 3) (48).

Unlike the *Streptomyces* enzyme, the dioxygenase from *Azotobacter* sp. strain GP1 exhibits a pronounced preference for nonchlorinated HQ. As in *Rhodococcus chlorophenolicus* (16), a reductive dechlorination of CHQ prior to *ortho* cleavage (Fig. 3) is conceivable, which allows an explanation of the preference of the *Azotobacter* enzyme for HQ as a substrate. No evidence, however, has been obtained to date for reductive dechlorination of CHQ in this organism. In the case of reductive dechlorination of CHQ as the main route for TCP metabolism in *Azotobacter* sp. strain GP1, one might expect a maleylacetate reductase which prefers maleylacetate over chloromaleylacetate. However, we found maleylacetate reductase activity which converts the chlorinated and the nonchlorinated substrates at comparable rates. Recent studies with maleylacetate reductases from *Alcaligenes eutrophus* JMP134 (38) and from *Pseudomonas* sp. strain B13 (20) have shown that the enzymes from both organisms catalyze two reactions, namely, the conversion of chloromaleylacetate to maleylacetate and the conversion of maleylacetate to 3-oxoadipate. As in *Azotobacter* sp. strain GP1, maleylacetate and chloromaleylacetate were converted with similar efficiencies. Therefore, our results with maleylacetate reductase reflect a typical feature of this enzyme and do not allow discrimination between the two possible pathways for the metabolism of CHQ.

Comparison of HQ 1,2-dioxygenase with other intradiol-cleaving dioxygenases. Catechol 1,2-dioxygenases catalyze the intradiol ring fission of 1,2-dihydroxybenzene compounds, yielding *cis-cis* muconates. The enzyme of this study catalyzes exactly the same type of reaction by cleaving 1,2,4-trihydroxybenzene between the hydroxy groups at positions 1 and 2. Surprisingly, this dioxygenase does not accept 1,2-dihydroxybenzene derivatives unless they bear a hydroxy group at position 4. This remarkably tight substrate specificity is shared by HQ 1,2-dioxygenase from *T. cutaneum* (40) and CHQ 1,2-dioxygenase from *S. rochei* 303 (48). Like the enzyme from

Azotobacter sp. strain GP1, the dioxygenase from *S. rochei* 303 was able to oxidize only HQ and CHQ; none of the other catechol, hydroquinone, and pyrogallol compounds tested served as a substrate. Table 5 presents substrate specificity data from additional HQ- and catechol-cleaving enzymes.

Catechol 1,2-dioxygenases can be divided into two types, I and II, according to Dorn and Knackmuss (10). Type I dioxygenases are relatively specific enzymes that use catechol primarily as a substrate. Chlorinated catechols are not used or are used only at negligible rates (29, 30). Type II enzymes are induced upon growth with a chlorinated carbon source and are relatively nonspecific with a wider substrate range. As shown in Table 5, they convert chlorinated catechols more rapidly than catechol and they accommodate additionally a wide range of, e.g., methyl- or methoxy-substituted catechols (6, 8, 10, 27, 39). With respect to substrate specificity, HQ 1,2-dioxygenases seem to be more closely related to the specific type I enzymes than to the chlorocatechol dioxygenases of type II. Unfortunately, HQ was not tested as a potential substrate for most catechol 1,2-dioxygenases; a catechol 1,2-dioxygenase which oxidized HQ at about half the rate (51%) that catechol did was described only for *T. cutaneum* (40).

The data for the molecular weights of the native enzymes and their subunits indicate that all of the HQ-cleaving dioxygenases and the representatives of catechol-cleaving dioxygenases are dimeric proteins with molecular masses of the same magnitude (Table 5).

Catechol- and chlorocatechol 1,2-dioxygenases are red or red-brown proteins containing 1 or 2 mol of ferric iron per mol of enzyme (6, 8, 30). Also, HQ 1,2-dioxygenase from *T. cutaneum* (40) and from *Azotobacter* sp. strain GP1 and the CHQ 1,2-dioxygenase from *S. rochei* (48) are colored iron proteins. X-ray fluorescence analysis revealed only about 0.2 mol of Fe in the *Azotobacter* enzyme. As shown in Table 4, the enzyme can be inhibited by the addition of iron-chelating agents like Tiron, and it can be significantly activated by the addition of iron. It should be mentioned that experiments to demonstrate activation by Fe²⁺ failed when phosphate buffer was used instead of Tris-HCl buffer, presumably because of the precipitation of iron phosphate. With CHQ 1,2-dioxygenase from *S. rochei* 303, Tiron (10⁻⁴ M) was shown to cause complete enzyme inhibition, which by addition of Fe²⁺ but not by Fe³⁺ could be restored to about 40% (47a). EPR spectra provide evidence for the presence of iron in the high-spin Fe(III) form and the absence of iron-sulfur components. This result coin-

cides with the inability to detect acid-labile sulfur in this protein. Obviously, the enzyme from *Azotobacter* sp. strain GP1 is a Fe(III)-containing dioxygenase similar to the other ortho-cleaving dioxygenases (6, 8, 30). Presumably, in the course of enzyme purification, iron is partially released from the active center, leading to a protein with a nonstoichiometric amount of Fe. It is interesting to note that Fe(II) ions are effective in enzyme activation although Fe(III) is in the prosthetic group. The conversion of Fe(II) in the presence of oxygen and the polyphenolic substrate to an Fe(III) enzyme species as postulated on the basis of EPR studies (31) might serve as an explanation. However, it should be pointed out that despite numerous spectroscopic, kinetic, and structural studies, the intimate mechanism of the enzymatic ring cleavage reaction remains unclear (8). As stated by Que (33), the spectroscopic data argue against the early mechanistic hypotheses which postulated a change in the valence state of iron contained in ring-cleaving dioxygenases during enzyme reaction.

Protein or DNA sequence analyses of various intradiol-cleaving dioxygenases have revealed a high degree of homology between chlorocatechol dioxygenases of type II (6, 12, 43). More-detailed genetic analyses indicate a common ancestral dioxygenase gene also with catechol 1,2-dioxygenases of type I (32, 43). The comparison of the NH₂-terminal amino acid sequence (40 residues) from HQ 1,2-dioxygenase from *Azotobacter* sp. strain GP1 revealed a relatively low degree of homology (between 7.5 and 22.5%) to catechol 1,2-dioxygenases from other organisms (6, 11, 12, 19, 23, 32, 43, 47). The highest homology value was found between HQ 1,2-dioxygenase from *Azotobacter* sp. strain GP1 and CHQ 1,2-dioxygenase from *S. rochei* 303 (48). Details of this result were already discussed by Zaborina et al. (48). However, whether the amino terminus is involved in metal or substrate binding and whether that partial sequence is relevant for making comparisons remain open.

The HQ 1,2-dioxygenases from *Azotobacter* sp. strain GP1 (this paper) and *S. rochei* 303 (13, 48) and the HQ 1,2-dioxygenase from *T. cutaneum* (40) catalyze the intradiol ring fission of a polyphenolic compound as do all other catechol 1,2-dioxygenases. Their inability to convert catechol compounds and their unique preference for 1,2,4-trihydroxybenzenes together with their elevated homology values in amino acid sequence as shown for the dioxygenases from *Azotobacter* and *Streptomyces* spp. suggest that these enzymes constitute a separate group among the other well-studied intradiol-cleaving dioxygenases.

ACKNOWLEDGMENTS

We are grateful to D. Küffer and H. Schreiber, Institute of Physics, University of Hohenheim, for performing X-ray fluorescence analysis, to R. Kappl, Institute of Biophysics, University of Saarland, Homburg, Germany, for EPR spectra, and to H. Weber and R. Wiesner, Fraunhofer Institute for Surface Technology and Biochemical Engineering, Stuttgart, Germany, for amino acid sequencing. We thank L. A. Golovleva, Russian Academy of Sciences, for supplying us with a sample of CHQ and M. Schlömann, Institute of Microbiology, University of Stuttgart, and W. Reineke, Institute of Chemical Microbiology, University of Wuppertal, for samples of butenolide and chlorobutenolide. The skillful fermentation performed by K. Kapassakalis is gratefully acknowledged.

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