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Metabolism of halohydroquinones in *Rhodococcus chlorophenolicus* PCP-1

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Abstract

The actinomycete *Rhodococcus chlorophenolicus* PCP-1 metabolises pentachlorophenol into ultimate inorganic end products via tetrachloro-*p*-hydroquinone. This intermediate was further dehalogenated in the cytoplasm requiring reductant in the cell free system. Tetrafluoro-*p*-hydroquinone and tetrabromo-*p*-hydroquinone were also dehalogenated. Chlorophenol analogs, thiol blocking agents and molecular oxygen inhibited the activity. The dehalogenating reactions led to 1,2,4-trihydroxybenzene, which was further metabolized into maleic acid.

Abbreviations: PCP – pentachlorophenol; TeCH – tetrachloro-*p*-hydroquinone; TeFH – tetrafluoro-*p*-hydroquinone; TeBH – tetrabromo-*p*-hydroquinone; THB – trihydroxybenzene

Introduction

Degradation pathways by actinomycetes are known for different haloaromatic compounds, such as chlorobenzoic acids, chlorinated phenols, chlorocinnamic acids and polychlorinated biphenyls. Strains capable of degrading chlorinated aromatic compounds have been found in several actinomycetal genera, such as *Mycobacterium, Rhodococcus, Arthrobacter, Corynebacterium, Brevibacterium, Nocardia* and *Streptomyces* (for a review see, Winter & Zimmermann 1992). The dechlorination of PCP involves *p*-hydroxylation with halohydroquinones as intermediates (Suzuki 1977; Apajalahti et al. 1986; Apajalahti & Salkinoja-Salonen 1986; Steiert & Crawford 1986; Apajalahti & Salkinoja-Salonen 1987a, 1987b; Häggblom et al. 1988, 1989; Schenk et al. 1989, 1990; Uotila et al. 1991; Golovleva et al. 1992; Uotila et al. 1992a, 1992b). *Rhodococcus chlorophenolicus* strain PCP-1 initiates PCP degradation by *para*-hydroxylation by a membrane bound cytochrome P-450 enzyme (Uotila et al. 1991, 1992a, 1992b). In some strains, such as *Rhodococcus, Streptomyces* and *Mycobacterium*, the product TeCH, is *ortho*-hydroxylated and the remaining chlorine atoms removed sequentially in reductive dechlorination steps by cytoplasmic enzymes (Apajalahti & Salkinoja-Salonen 1987b; Häggblom et al. 1989; Uotila et al. 1992b). The product of this dechlorination sequence was identified as nonhalogenated 1,2,4-THB (Apajalahti & Salkinoja-Salonen 1987b; Uotila et al. 1992b). The aim of the present study was to characterise the downstream metabolism of TeCH and 1,2,4-THB. We describe the substrate specificity, sensitivity to inhibitors and the requirements for activity of the TeCH-dechlorinating activity. Evidence for the channelling of the resulting 1,2,4-THB into the intermediary metabolism of *R. chlorophenolicus* PCP-1 via maleic acid is shown.

Materials and methods

Bacterial strains, culture conditions and chemicals

Rhodococcus chlorophenolicus PCP-1 (DSM 43826, Apajalahti et al. 1986) was grown and induced with PCP as described by Uotila et al. (1991). Sources and/or methods for synthesis of the authentic chlorinated reference compounds and THBs were as described earlier by Apajalahti and Salkinoja-Salonen (1987a, 1987b) and Häggblom et al. (1988, 1989). TeFH and TeBH were purchased from Tokyo Kasei (Tokyo, Japan). The inhibitors tested were purchased as follows: diisopropylfluorophosphate, menadione and metyrapone (Sigma, St. Louis, MO, USA); Tiron (Siegfried, Switzerland); 8-hydroxyquinoline, 2,2′-dipyridyl and $HgCl_2$ (Meck, Darmstadt, Germany); methylenebisthiocyanate (Riedel-de Haen, France); 4-chloromercuribenzoate (Fluka AG, Buchs, Switzerland); SKF-525A was a kind gift of Smith Kline & French Labs Ltd., UK; parathion (Labor Dr. Ehrenstorfer, Augsburg, Germany). Bio-Rad reagent for protein determination was purchased from Bio-Rad Laboratories (Richmond, CA, USA); egg white lysozyme and gel filtration molecular weight markers MW-GF-200 (Sigma, St. Louis, MO, USA); succinic acid, fumaric acid and maleic acid (Fluka AG, Buchs, Switzerland); FPLC columns, preparative isoelectric focussing kit (Ampholine pH 3.5–10), SDS-PAGE molecular weight calibration kit, SDS-PAGE equipment (2050 Midget gel apparatus, 10% gel) from Pharmacia LKB (Uppsala, Sweden). Deoxyribonuclease I was from Boehringer-Mannheim (Mannheim, Germany). Ultrafee-Cl Low Binding Cellulose was from Millipore (Nihon Millipore Kogyo K.K., Yonezawa, Japan). All the buffers and chemicals used were freshly made in Milli-Q water (Milli-Q plus, Millipore, Molsheim, France).

Partial purification of the TeCH dehalogenating activity

Five litres of the culture were harvested by centrifugation (3000 × g, 10 min, 4° C), the cells resuspended (1 g wet wt ml^{-1}) in borax-buffer (50 mM, pH 8.0, Sober 1973) and disrupted by French press at 4.8 × 10^5 Pa. Deoxyribonuclease I was added to the extract (50 $\mu g\ ml^{-1}$), after one hour at 22° C the unbroken cells were removed by centrifugation (3000 × g, 10 min, 4° C) and the supernatant centrifuged at 150 000 × g, for 90 min, 4° C. The pellet was discarded and the supernatant (the soluble proteins, specific activity 0.022 nkat mg^{-1} protein) used for analyses. Ammonium sulphate was added to 65% of saturation to the 150 000 × g supernatant of the cell extract and the precipitate formed was collected after 20 min by centrifugation at 20 000 × g for 20 min at 4° C. The precipitate was dissolved in 2 ml of borax buffer and washed three times with 2 ml of the same buffer (specific activity 0.044 nkat mg^{-1} protein). Excess ammonium sulphate was removed using Millipore Ultrafee-Cl Low Binding Cellulose. The desalted precipitate was fractionated by a Q-Sepharose HP HiLoad 26/10 anion exchange column, using 50 mM borax buffer (pH = 8.0) and a NaCl gradient from 0 to 1 M at a flow rate of 2 ml/min. The fractions containing TeCH consuming activity (eluted at 0.25–0.3 M NaCl, specific activity 1.2 nkat mg^{-1} protein) were pooled, concentrated with Millipore Ultrafee-Cl Low Binding Cellulose and further fractionated by a Superose-12 HR 10/30 gel permeation chromatography column equilibrated and eluted with borax (0.3 ml/min). The TeCH comsuming fractions were pooled and the pooled fraction was

Table 1. Effect of substrate analogues on TeCH turnover by the pooled GPC fraction.

Substrate analog*	Relative substrate removal*
None	100%
Trichlorohydroquinone	60%
2,3-dichlorohydroquinone	40%
2,5-dichlorohydroquinone	100%
2,6-dichlorohydroquinone	100%
PCP	18%
2,3,4,5-tetrachlorophenol	30%
2,3,4,6-tetrachlorophenol	60%
2,3,5,6-tetrachlorophenol	5%
4-methoxytetrachlorophenol	93%
1,4-dimethoxytetrachlorobenzene	100%
1,2,3-THB	80%
1,2,4-THB	90%
1,3,5-THB	70%

* The assay for the substrate turnover contained partially purified TeCH dehalogenating GPC fraction (12 $\mu g\ ml^{-1}$ of protein), substrate (50 μM), substrate omalogue (50 μm) and ascorbic acid (1 $mg\ ml^{-1}$). For TeCH the 100% turnover was 5.2 μmol of TeCH h^{-1} mg $protein^{-1}$. The TeCH removal was calculated on the linear reaction time (3 h).

Table 2. Effect of enzyme inhibitors on TeCH dehalogenating pooled GPC fraction.

* Inhibitor	Concentration	* Relative substrate removal
None	–	100%
Serine enzyme inhibitor:		
Diisopropylfluorophosphate	5 mM	100%
Cation chelators:		
Tiron	5 mM	97%
8-Hydroxyquinoline	5 mM	85%
2,2′-Dipyridyl	5 mM	54%
Methylenebisthiocyanate	5 mM	5%
Thiol blocking agents:		
$HgCl_2$	5 mM	20%
4-Chloromercuribenzoate	5 mM	9%
P-450 inhibitors:		
Metyrapone	5 mM	93%
SKF-525A	5 mM	14%
Menadione	5 mM	9%
Parathion	5 mM	80%
CO (with dithionite)	(pCO = 1.0)	100%
	(pCO = 0.1)	100%
Gases:		
O_2	(pO_2 = 1.0)	67%
Ar	(pAr = 1.0)	100%

* The assay for the substrate turnover contained partially purified TeCH dehalogenating GPC fraction (12 μg ml^{-1} of protein), TeCH (50 μM), inhibitor and ascorbic acid (1 mg ml^{-1}). For TeCH the 100% turnover was 5.2 μmol of TeCH h^{-1} mg protein^{-1}. The TeCH removal was calculated on the linear reaction time (3 h).

Table 3. Effect of various reductants on the turnover of TeCH by the pooled GPC fraction.

* Reductant E_h °	* TeCH removal (μmol h^{-1} protein mg^{-1}) GPC fraction present	
	No	Yes
No reductant	< 0.05	0.3
Ascorbic acid (- 12 mV)	< 0.05	5.2
Cysteine (- 182 mV)	< 0.05	9.4
Glutathione (- 230 mV)	< 0.05	9.1
NADH (- 320 mV)	< 0.05	6.6
NADPH (- 320 mV)	< 0.05	11.9

* The assay for the substrate turnover contained partially purified TeCH dehalogenating GPC fraction (12 μg ml^{-1} of protein), TeCH (50 μM) and a reductant (100 μM). The TeCH removal was calculated on the linear reaction time (3 h).

used in all assays (specific activity 4.3 nkat mg^{-1} protein).

Analyses

TeCH dechlorinating activity was assayed with 50 or 100 μM of substrate. protein was determined by the method of Bradford (1976) using egg white lysozyme for calibration. The active enzyme fractions were analysed by SDS-PAGE using the method of Laemmli (1970). The silver staining was performed according to Ohsawa and Ebata (1983). The rates of substrate turnover (Tables 1, 2 and 3) were determined by gas liquid chromatography at the linear reaction time (3 h) as described earlier (Apajalahti & Salkinoja-Salonen 1986; Uotila et al. 1991) using the internal standard method. Ascorbic acid (1 mg ml^{-1}) was added to the buffers used for activity assays (Apajalahti & Salkinoja-Salonen 1987b). The metabolites emerging from halohydroquinones were analysed as trimethylsilyl derivatives with gas liquid chromatography mass spectrometry (HP 5890A gas chromatograph with 5970 mass selective detector) operating in total ion monitoring mode as described earlier (Apajalahti et al. 1987a, 1987b; Uotila et al. 1992a, 1992b). Metabolites of 1,2,4-THB were analysed as follows: 150 000 × g supernatant (70 mg of protein) was diluted into 1000 ml of borax bufer containing ascorbic acid (1 mg ml^{-1}) and 1,2,4-THB (100 μM). After incubating at 37° C for 1 h, the reaction was stopped by dropping the pH to 1 by addition of HCl. The metabolites were extracted twice with 200 ml of diethyl ether, extracts combined, dried with dehydrated Na_2SO_4 and evaporated in a rotating evaporator. The dried residue was methylated with diazomethane (Schlenk & Gellermann 1960). The derivatized metabolites were analysed by gas liquid chromatography mass spectrometry in total ion monitoring mode using splitless injection and a temperature program from 70° C to 200° C at 15° C/min, 200° C for 1 min and 5° C/min to 290° C. A reference assay was prepared similarly using extract from cells grown in the absence of chlorinated phenolic compounds.

Inhibition assays

The TeCH dehalogenating fraction (12 μg ml^{-1} of protein) was incubated in the presence of an inhibitor (5 mM), substrate (50 μM) and ascorbic acid (1 mg

ml^{-1}) at 37° C for 3 h. The effects of 50 μM substrate analogues were tested similarly. Abiotic removal of TeCH was followed in parallel vials containing no enzyme. The requirement for electron donor (ascorbic acid, cysteine, glutathione, NADH and NADPH 100 μM) was tested under anoxic conditions to minimise autooxidation of TeCH. The TeCH consuming fraction (12 $\mu g\ ml^{-1}$ of protein) and reductant (1 mg ml^{-1}) were sealed in a gas tight ampoule and the oxygen removed by repeated cycles of evacuation and purging with argon (99.9995%). A deoxygenated solution of TeCH (final concentration 100 μM) was injected into the ampoule to start the reaction. The reaction was allowed to proceed at 37° C for 3 h, and stopped with an injection of the acetylation reagents (Apajalahti & Salkinoja-Salonen 1986). Oxic and anoxic conditions were separately tested by adjusting the partial pressure of either oxygen (99.995%) or argon (99.9995%) to 0.1 or 1.0 in the head space of the ampoules.

Results

TeCH dehalogenating activity

TeCH consuming activity of cell extracts of PCP-induced *Rhodococcus chlorophenolicus* PCP-1 was purified by ultracentrifugation, ammonium sulphate precipitation, ion exchange chromatography and gel permeation chromatography. The TeCH consuming activity eluted from Superose 12 HR 10/30 column as a single peak, corresponding to 102 kD based on the gel permeation chromatography calibration used. The specific TeCH consuming activity of this fraction was 200 fold compared with the crude extract, the final specific activity being 4.3 nkat mg^{-1} of protein. The subsequent SDS-PAGE of the TeCH active fraction showed 10 bands of similar intensity ranging from 14 kD to 66 kD. When the active fraction was digested with proteinase K (1 h, 1 mg ml^{-1}, in borax buffer), the TeCH dechlorinating activity was lost, showing that the activity was mediated by a protein. Further purification was attempted using sequential combinations of thiol Sepharose 4B, phenyl Superose HR 5/5, Mono-Q HR 5/5, Mono S HR 5/5, Mono P HR 5/5 and preparative isoelectric focussing, but no further increase of specific activity was obtained. The activity was lost after thiol Sepharose 4B, Mono S HR 5/5, Mono P HR 5/5 and preparative isoelectric focussing and the activity did not reappear after combining the fractions. The presence of dithiothreitol and β-mercaptoethanol did not restore the activity. The TeCH consuming activity was stable, with retention of 90% of the activity after storage for three weeks at + 4° C. One explanation for the failure of further purification could be that several proteins contribute to the activity.

*Metabolites emerging from tetrahalo-*p*-hydroquinones and 1,2,4-trihydroxybenzene*

TeFH and TeBH were tested as substrates of the TeCH consuming pooled GPC fraction. The reaction products obtained from TeFH and TeBH were analysed by gas liquid chromatography mass spectrometry. It was found that the nonhalogenated product, 1,2,4-THB based on its mass spectra and retention time, was not only produced from TeCH, but also from TeFH and TeBH (Fig. 1). Treatment with proteinase K (1 h, 1 mg ml^{-1}, in borax buffer) destroyed also the TeFH and TeBH consuming activity. The pooled TeCH consuming GPC fraction produced dichlorotrihydroxybenzene, and monochlorotrihydroxybenzene as the intermediates from the dehalogenation of TeCH as described earlier (Apajalahti & Salkinoja-Salonen 1987b). Corresponding fluorinated (mass fragments 381 and 363) and brominated (mass fragments 503 and 423) trihydroxybenzenes were found when TeFH and TeBH were incubated with the active fraction. This fraction therefore dehalogenated halohydroquinones with di- and monohalogenated trihydroxybenzenes as intermediates.

A new peak was found by gas liquid chromatography mass spectrometry in the total ion chromatograms when 150 000 × g supernatant was incubated with 1,2,4-THB. The m/e of the relevant mass fragments and retention time of the emerging peak were similar to those of dimethylester of authentic maleic acid (Fig. 2). In the control experiments where either 1,2,4-THB substrate or the 150 000 × g supernatant was omitted, no dimethylester of maleic acid was found, indicating that the maleic acid originated from 1,2,4-THB and its formation was mediated by the proteins in the 150 000 × g supernatant. When 150 000 × g supernatant was prepared using extract from cells grown in the absence of chlorinated phenolic compounds, the indicative mass fragments of dimethylester of maleic acid were not observed (detection limit: 1/10 of the induced level), suggesting that the 1,2,4-THB cleaving enzymes were inducible.

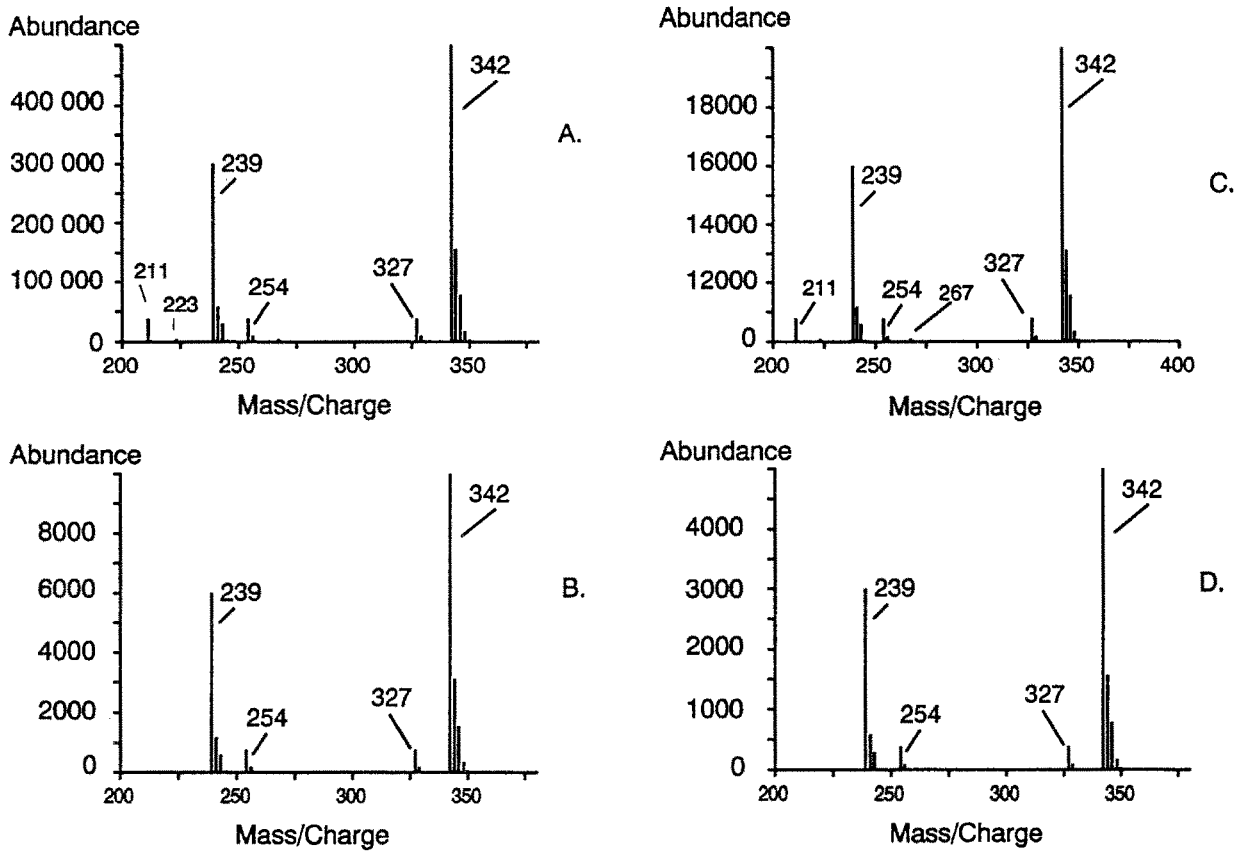

Fig. 1. Mass fragmentation patterns of the authentic silylated model compound 1,2,4-THB and silylated metabolites formed from each halogenated hydroquinone. (A) Authentic 1,2,4-THB. (B) Product from TeCH. (C) Product from TeFH. (D) Product from TeBH.

Substrate specificity

The turnover of a number of haloaromatic compounds by the TeCH dehalogenating pooled GPC fraction were tested. TeFH (4.8 μmol of TeFH h^{-1} mg $protein^{-1}$) and TeBH (3.6 μmol of TeBH h^{-1} mg $protein^{-1}$) were dehalogenated with a rate similar to that of TeCH (5.2 μmol of TeCH h^{-1} mg $protein^{-1}$). The turnover rate of trichlorohydroquinone was low (0.2 μmol of trichlorohydroquinone h^{-1} mg $protein^{-1}$) compared to TeCH and nondetectable for the 2,3-, 2,5- and 2,6-dichlorohydroquinones. PCP, tetrachlorocatechol, tetrachlororesorcinol and methoxylated derivatives of TeCH (4-methoxytetrachlorophenol and 1,4-dimethoxytetrachlorobenzene) were not converted. The effect of incubating the TeCH consuming pooled GPC fraction with various substrate analogues (50 μM) on the turnover of TeCH (50 μM) was tested (Table 1). It was found that, TeCH removal was halved when either trichlorohydroquinone or 2,3-dichlorohydroquinone was present. Tetra- and pentachlorophenols inhibited dechlorination of TeCH even more strongly: inhibition by 2,3,5,6-tetrachlorophenol was close to 100% (Table 1). THBs, 4-methoxytetrachlorophenol and 1,4-dimethoxytetrachlorobenzene did not affect the dechlorination of TeCH.

Oxygen dependence and effect of enzyme inhibitors on the TeCH turnover

Replacing the air from the reaction vessel by pure oxygen inhibited TeCH turnover somewhat, but full activity was retained when air was replaced by argon or carbon monoxide (with dithionite to remove dissolved oxygen). It thus seems that hydrolytic oxygen incorporated into TeCH must have been derived from water rather than molecular oxygen. To further characterise the halohydroquinone dehalogenating activity, we tested the effect of enzyme inhibitors on the pooled GPC fraction (Table 2). The divalent cation chelators methylenebisthiocyanate and 2,2-dipyridyl

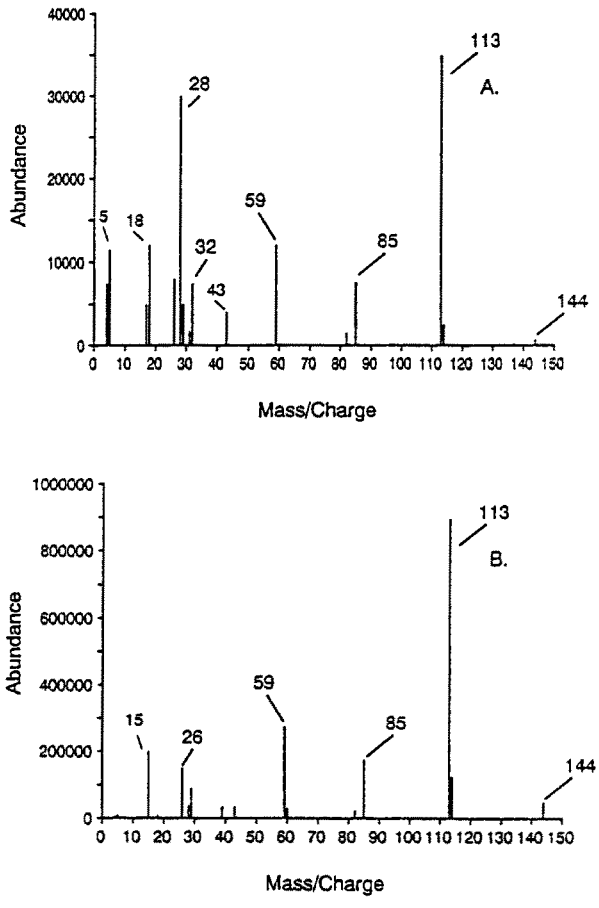

Fig. 2. (A) Mass spectrum of the methylated metabolite from 1,2,4-THB. (B) Mass spectrum of the methylated authentic maleic acid.

inhibited the dechlorination of TeCH, while ferric iron chelating agents, Tiron and 8-hydroxyquinoline, had no effect. Thiol group blocking agents, $HgCl_2$ and 4-chloromercuribenzoate strongly inhibited the turnover of TeCH. Addition of reducing agent, β-mercaptoethanol also inhibited the turnover of TeCH for 65% (data not shown). Diisopropylfluorophosphate, a serine enzyme inhibitor, did not affect TeCH turnover. The inhibitors of cytochrome P-450 showed diverse action: menadione and SKF-525A inhibited the reaction strongly, but metyrapone, parathion and carbon monoxide (with dithionite) did not. The TeCH dehalogenating activity was therefore inhibited by 2,2′-dipyridyl and thiol group blocking agents, but not by typical P-450 inhibitors.

Requirement for reducing power of the TeCH consuming pooled GPC fraction

We tested the effect of several reductants on the turnover of TeCH under anoxic conditions, under which nonenzymatic oxidation of TeCH was not significant. Table 3 lists the effects of various reductants, and their E_h(mV) values. All of the five hydrogen donors tested (100 μM), ascorbic acid, cysteine, glutathione, NADH and NADPH, stimulated turnover of TeCH. The TeCH turnover rates did not correlate with the oxidation-reduction potential of the reductant. Highest activity, 11.2 μmol of TeCH h^{-1} mg^{-1} protein, was obtained with NADPH (Table 3). In the absence of reductant the activity was 40 times lower (0.3 μmol of TeCH h^{-1} mg^{-1} protein). None of the reductants catalyzed TeCH turnover in the absence of the TeCH active cell fraction. The TeCH dehalogenating activity thus had a non specific requirement for reductant.

Discussion

Reductive aryl dehalogenation reactions, where halogen is substituted by hydrogen, have been reported in a few aerobic bacteria (Apajalahti & Salkinoja-Salonen 1987b, Häggblom et al. 1989, Uotila et al. 1992b, Xun et al. 1992a), but the enzymes involved in this catalysis are not generally well known. In this paper we report on the properties of a hydroxylating and reductive dehalogenating activity for halohydroquinones in an aerobic bacterium, *R. chlorophenolicus* PCP-1. Aerobic PCP degraders, *Corynebacterium* sp. KC-3, and *Flavobacterium* sp. and *R. chlorophenolicus* PCP-1 (Reiner et al. 1978, Steiert & Crawford 1986, Apajalahti & Salkinoja-Salonen 1987a, 1987b), were shown to remove at least three of the chlorines before the ring cleavage. We earlier demonstrated the conversion of chlorinated, fluorinated and brominated hydroquinones into 1,2,4-THB by soluble enzymes of *Mycobacterium fortuitum* CG-2 (Uotila et al. 1992b). This report shows that soluble proteins from *R. chlorophenolicus* PCP-1 cell extract dechlorinated, defluorinated and debrominated the tetrahalo-*p*-hydroquinones via di- and monohalogenated trihydroxybenzenes into the same central intermediate 1,2,4-THB. Chapman and Ribbons showed (Chapman & Ribbons 1976a, 1976b) that the cleavage of the aromatic ring of 2,3,5-trihydroxytoluene in *Pseudomonas putida* was mediated by two different hydroxyhydroquinol 1,2-dioxygenase activities pro-

ducing a maleylacetate or 2,4,6-trioxoheptanoate. 2,4-dihydroxy-6-oxo-2,4-hexadienoic acid was proposed in *Rhodococcus* sp. BPG-8 (Armstrong & Patel 1992) and maleylacetate in *Streptomyces rochei* 303 (Golovleva et al. 1992) as intermediates in the ring cleavage of trihydroxybenzene. In this paper we showed that cleavage of 1,2,4-THB yielded maleic acid in cell extracts of *R. chlorophenolicus* PCP-1. We also showed that this cleavage was an inducible activity. Information on aliphatic halidohydrolases has been accumulating (Kawasaki et al. 1981a, 1981b; Walker et al. 1981; Motosugi et al. 1982a, 1982b; Keuning et al. 1985; Estrada Diaz et al. 1989; Kocabiyik & Turkoglu 1989; Van den Wijngaard et al. 1991). These enzymes mostly have an alkaline pH optimum and optimum temperature between 45° C and 55° C. Furthermore, most are sensitive to thiol blocking agents such as $HgCl_2$ and 4-chloromercuribenzoate, indicating that sulfhydryl groups may have a role in the enzyme function. Our results indicate that the halohydroquinone dehalogenating activity in *R. chlorophenolicus* PCP-1 was similar to the aliphatic halidohydrolases, having a pH optimum of 8, temperature optimum of 50° C, sensitivity to sulfhydryl blocking agents and a preference for anaerobic conditions (Apajalahti & Salkinoja-Salonen 1986, 1987a, 1987b and this paper). The hydroxylating and reductive dehalogenation of TeCH by *R. chlorophenolicus* PCP-1 (Apajalahti & Salkinoja-Salonen 1987a, 1987b; Uotila et al. 1991, 1992a) extracts was insensitive to Fe^{2+}-chelating compounds. Methylenebisthiocyanate did inhibit TeCH dehalogenation, but this may have been caused by its hydrolysis product, formaldehyde, formed at pH 8 (McCoy 1980). Therefore the hydroxylation reaction of TeCH probably is different from the mechanism described by Castro et al. (1985) for reductive dehalogenation of trichloronitromethane in *Pseudomonas putida*. In this paper we show that the same typical P-450 inhibitors (Testa & Jenner 1981), which blocked *para*-hydroxylation of PCP in *R. chlorophenolicus* PCP-1 extracts (Uotila et al. 1991) had no effect on the TeCH dehalogenation. This finding indicates that regular cytochrome P-450 type of enzymes are unlikely to be involved. The halohydroquinone dehalogenase of *R. chlorophenolicus* PCP-1 had nonspecific requirement for reducing power and was more active in the absence than in the presence of molecular oxygen. Menadione inhibited activity but this may be explained by the bleed of the reductant required for the enzymatic reactions. Xun et al. (1992a, 1992b) showed a requirement for reductant in cell extracts and purified TeCH reductive dehalogenase of *Flavobacterium* sp. strain ATCC 39723 attacking TeCH, but in their case reduced glutathione was the only accepted reductant. A cell extract of *Clostridium rectum* was shown to dechlorinate lindane under anaerobic conditions, also nonspecifically requiring for reducing power: dithiothreitol, NADH and NADPH were accepted as electron donors (Ohisa et al. 1980). Thus the TeCH dehalogenase of *R. chlorophenolicus* PCP-1 may resemble more *Clostridium* dehalogenase than that of the *Flavobacterium* sp.

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