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Dechlorination of pentachlorophenol by membrane bound enzymes of *Rhodococcus chlorophenolicus* PCP-I

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Abstract

Dechlorination (*para*-hydroxylation) of pentachlorophenol (PCP) and tetrachloro-*para*-hydroquinone (TeCH) and O-methylation of TeCH were demonstrated in cell extracts of *Rhodococcus chlorophenolicus* PCP-I. PCP *para*-hydroxylating activity was membrane bound, whereas TeCH dechlorinating enzyme was soluble. The PCP *para*-hydroxylating enzyme was solubilized by Triton X-100 and the requirement for both FAD and NADPH was shown. The dechlorinating activities were inducible in contrast to the constitutive TeCH O-methylating activity. The PCP *para*-hydroxylation was inhibited by its product TeCH, by anoxic conditions, and by different inhibitors of P_{450}. Participation of this cytochrome in the PCP hydroxylation was confirmed by the appearance of a carbon monoxide dependent peak of absorbance at 457 nm in the membrane fraction prepared from PCP degrading cells.

Introduction

Enzymic reactions involved in microbial removal of halogen atoms from polychlorinated aromatic compounds are only partially understood. Dechlorination of pentachlorophenol (PCP) in a cell free system has been described by Schenk et al. (1989) and dechlorination of tetrachloro-*para*-hydroquinone (TeCH) by Apajalahti & Salkinoja-Salonen (1987b).

Rhodococcus chlorophenolicus PCP-I (Apajalahti & Salkinoja-Salonen 1986a) dechlorinates many different polychlorinated phenolic compounds (Apajalahti & Salkinoja-Salonen 1986b, 1987a; Häggblom et al. 1988a). The degradation of chlorophenols by this bacterium proceeds via sequential hydroxylating and reductive removal of chlorine substituents. The product of the dehalogenating reactions is 1,2,4-trihydroxybenzene, which is converted eventually to CO_2 (Apajalahti & Salkinoja-Salonen 1987a, b).

R. chlorophenolicus PCP-I dechlorinates PCP by inducible enzymes into TeCH (Apajalahti & Salkinoja-Salonen 1987a). TeCH is further dechlorinated by an inducible, soluble enzyme complex described earlier by Apajalahti & Salkinoja-Salonen (1987b). Cells grown in the absence of chlorophenols methylate TeCH into a dead end product, tetrachloro-*para*-methoxyphenol.

In this paper we show that the *para*-hydroxylation of PCP is catalyzed by a membrane associated enzyme of *R. chlorophenolicus* PCP-I and involves cytochrome P_{450} type coenzymes.

Materials and methods

Bacterial strains and culture conditions

Rhodococcus chlorophenolicus PCP-I (DSM 43826; Apajalahti et al. 1986a) was grown in nutrient broth yeast extract (NBYE)-medium (Singer & Finnerty 1988) in a gyratory shaker at 28° C. Two-days grown cultures were induced by adding increasing amounts (10 to 50 μM) of PCP or TeCH at 24 h intervals.

Cell fractionation

Cells were harvested by centrifugation (3000 × g, 10 min, 4° C) and resuspended (1 g wet wt ml^{-1}) in borax-buffer (50 mM $Na_2B_4O_7$, pH = 8.0). Cells were disrupted by French press at 6000 PSI, and deoxyribonuclease I was added to the cell extract (50 μg ml^{-1}, Boehringer-Mannheim, Germany). After 1 h at 22° C the unbroken cells were removed by centrifugation (3000 × g, 10 min, 4° C) and the supernatant further centrifuged at 150000 × g, for 90 min, 4° C. The pellet was washed once with 10 volumes of the borax-buffer, and resuspended into the original volume of the same buffer. The 150000 × g pellet was solubilized by resuspending it into the borax-buffer containing 0.1% (vol/vol) of Triton X-100.

Analyses

For activity assays 1.9 mg ml^{-1} of protein and 20 to 50 μM substrate (PCP or TeCH) were used. Protein was determined by the method of Bradford (1976) with Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA), using egg white lysozyme for calibration (Sigma, St. Louis, MO). Assay for PCP dechlorination was carried out in the borax buffer at pH 8.0. In the assay for TeCH dechlorination ascorbic acid (1 mg ml^{-1}) was added to prevent abiotic oxidation of TeCH. The enzymatic activity was calculated over time period of linear activity (Hh).Reaction mixture with no enzyme served as the blank.

Chlorophenolic compounds were acetylated and analyzed by gas liquid chromatography (GLC) using internal standard method as described earlier (Apajalahti & Salkinoja-Salonen 1986b). A Sil 19 CB capillary column (Chrompack International B.V., Middelburg, The Netherlands) was used. Metabolites emerging were identified by GLC-MS as described by Apajalahti & Salkinoja-Salonen 1987a, b).

Tetrachloro-*para*-methoxyphenol was prepared by partially methylating TeCH with $(CH_3)_2SO_4$ in alkaline environment (Knuutinen & Kolehmainen 1983).

Inhibition assays

For the anoxic assays the reaction mixture was enclosed in gas tight vials, and flushed with argon (99.998%) for 30 min. Deoxygenated substrate was injected into the vial to start and the acetylation reagents to stop the reaction. The amount of dissolved oxygen was measured with an Orion Research oxygen electrode Model 97-08-99.

Inhibitors tested were Tiron (5 mM, Siegfried S.A., Switzerland), methylenebisthiocyanate (5 mM, Riedel-de Haen, FRG), Metyrapone (5 mM, Sigma), menadione (5 mM, vitamin K_3, prereduced, Sigma) and SKF-525A (5 mM, Smith Kline & French Labs, UK).

Inhibition by carbon monoxide was tested by adjusting its partial pressure to 0.1 or 0.01 in gas tight vials under air.

Spectrophotometric analysis

Absorption spectra were measured at room temperature using the single beam mode of a Shimadzu UV-3000 spectrophotometer. The proteins of 150000 pellet (4.2 mg in 0.5 ml in borax buffer) were solubilized with 1% (v/v) Triton X-100. Sodium dithionite (10 mM) was added to create reducing environment and a spectrum (400–500 nm) was measured before and after purging with carbon monoxide.

The concentration of the cytochrome P_{450} was

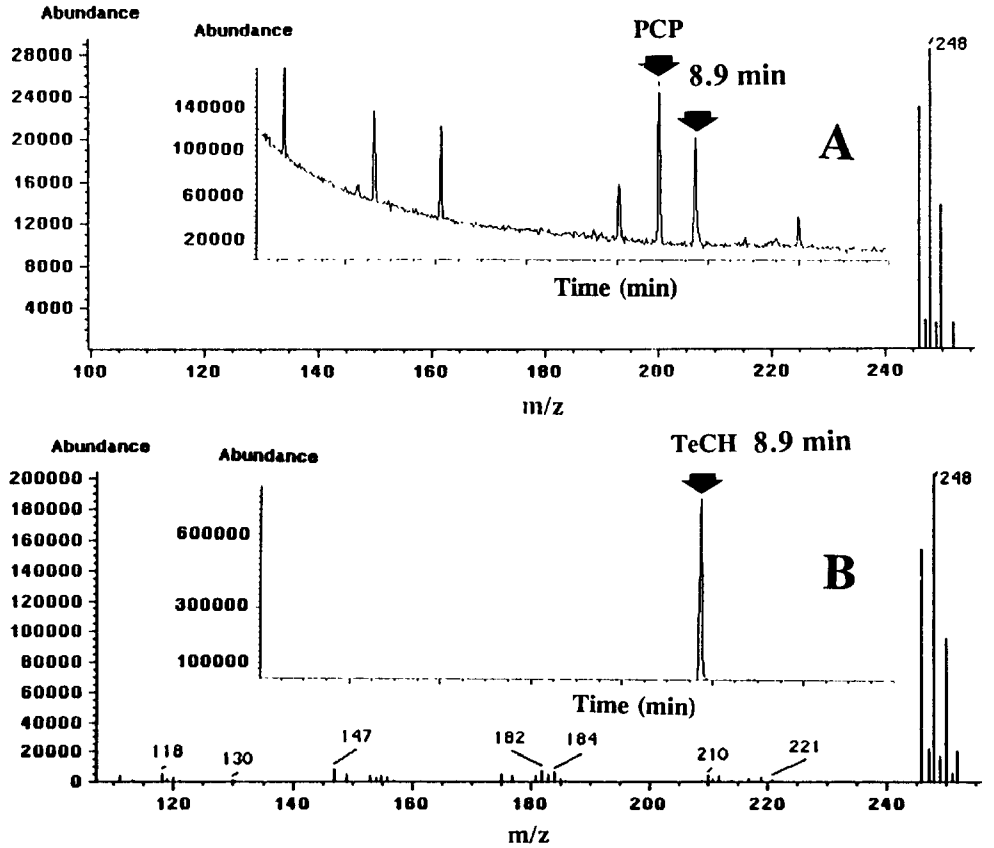

Fig. 1. Mass spectra of the metabolite formed from PCP by 150000 × g pellet of *R. chlorophenolicus* PCP-I (Fig. A) and of authentic TeCH (Fig. B). The corresponding total ion chromatograms are shown in the respective inserts (Acetyl derivatives).

calculated from the reduced carbon monoxide differential spectrum, using the specific extinction coefficient of *Pseudomonas putida* enzyme, $\blacktriangle A_{450-490} = 92.8\,cm^{-1}\,mM^{-1}$ (Gunsalus & Wagner 1978).

Results

Dechlorinating activities in cell extracts of R. chlorophenolicus *PCP-I*

Table 1 shows the dechlorination activities of the extracts prepared from PCP-induced and TeCH-induced cells of *R. chlorophenolicus* PCP-I. The turnover rate of PCP by the crude extract from PCP-induced cells was 1.75 nmol h^{-1} mg $protein^{-1}$. A new peak was observed in the gas chromato-

Table 1. Turnover of PCP and TeCH by cell extracts from *Rhodococcus chlorophenolicus* PCP-I.

Substrate	Specific activity of (nmol substrate h^{-1} mg $protein^{-1}$)**		
	Crude extract	Supernatant*	Pellet*
From uninduced cells			
PCP	0.05	0.05	0.05
TeCH	1.3 (1.3)	1.7 (1.7)	0.05 (0.05)
From PCP-induced cells			
PCP	1.75	0.19	2.2
TeCH	12.5 (<0.05)	13.3 (<0.05)	4.3 (<0.05)
From TeCH-induced cells			
PCP	0.1	0.08	0.05
TeCH	5.6 (<0.05)	5.5 (<0.05)	4.3 (<0.05)

* Centrifuged 90 min at 150000 × g.

** The figures in the parenthesis show the rate of tetrachloro-*para*-methoxyphenol formation in the assays.

gram, the retention time and the mass spectrum of which were identical to those of authentic TeCH (Fig. 1). PCP thus had undergone dechlorination and *para*-hydroxylation. The reaction rate was highest at pH = 7.5. Extracts prepared from uninduced and from TeCH-induced cells showed little or no activity towards PCP (Table 1), indicating that the *para*-hydroxylating activity was induced by PCP and not by TeCH.

TeCH was consumed by the extract from PCP-induced cells (12.5 nmol TeCH h^{-1} mg $protein^{-1}$), the turnover rate being higher than that of the PCP. Induction with TeCH was less effective, possibly because of the abiotic oxidation of TeCH when added to the aerobic culture. The extract converted TeCH at the rate of 5.6 nmol h^{-1} mg $protein^{-1}$.

When the cell extract was fractionated by centrifugation (150000 × g), the PCP *para*-hydroxylating activity was retained in the 150000 × g pellet (2.2 nmol PCP h^{-1} $protein^{-1}$). TeCH-consuming activity remained mainly in the supernatant (13.3 nmol TeCH h^{-1} mg $protein^{-1}$, Table 1), which contained 73% of the cell extract protein.

There was a TeCH-removing activity also in extracts prepared from uninduced cells, but in these the consumption of TeCH was parallel and stoichiometric to the accumulation of a metabolite, which was identified in GLC-MS as tetrachloro-*para*-methoxyphenol. This activity was not found in the extracts of chlorophenol-induced cells (Table 1).

Table 2. The effects of various cofactors on PCP *para*-hydroxylation in particulate and solubilized 150000 × g pellets from *R. chlorophenolicus* PCP-I.

Cofactor	Specific activity of (nmol substrate h^{-1} mg $protrein^{-1}$)	
	pellet*	pellet* dissolved in Triton X-100
None	2.20	0.25
FAD	2.10	< 0.05
NADPH	2.25	0.05
FAD + $NADP^+$	2.20	0.05
FAD + NADPH	2.25	2.60

* Prepared from PCP-induced cells by centrifugation at 150000 × g.

PCP para-*hydroxylation by solubilized membranes*

The PCP *para*-hydroxylating activity was lost upon dissolving the membrane fraction in detergent (Table 2). The abilities of $NADP^+$ (1 mM); NADPH (1 mM) and FAD (1 mM) to restore PCP degradation by the 150000 × g pellet, are also shown in Table 2. The activity was restored by the addition of FAD + NADPH, but not by FAD + $NADP^+$. None of the tested cofactors had effect on the activity of 150000 × g pellet in the absence of detergent.

Inhibition of PCP para-*hydroxylation*

Dechlorination of PCP by the cell extract of *R. chlorophenolicus* PCP-I was totally inhibited by the product, TeCH (50 μM), when added together with 20 μM PCP (Table 3). The cytochrome P_{450} inhibitors SKF 525A, metyrapone and menadione inhibited *para*-hydroxylation of PCP (Table 3). Anoxic conditions had the same effect (argon), but the activity was totally recovered when oxygen was reintroduced into the vials after 24 h of anoxic incubation. Oxidized coenzymes (FAD^+, $NADP^+$ did not restore the activity in the anoxic vial.

Table 3. Effect of potential inhibitors on PCP dechlorinating activity by the 150000 × g pellet from PCP-induced *R. chlorophenolicus*.

Test conditions		Specific activity of (nmol substrate h^{-1} $protein^{-1}$)
No inhibitor present		2.20
TeCH	(50 μM)	< 0.05
SKF-525A	(5 mM)	< 0.05
Metyrapone	(5 mM)	0.50
Menadione	(5 mM, saturated)	0.40
Anoxic condition:		< 0.05*
Sodium dithionite (5 mM):		
ambient air		1.95
P_{co} = 1.0		< 0.05
P_{co} = 0.1		0.65
P_{co} = 0.01		1.90
Tiron	(5 mM)	2.10
Methylenebisthiocyanate	(5 mM)	0.50

* Reintroduction of air after 24 h of incubation under anoxic conditions led to recovery of 100% activity.

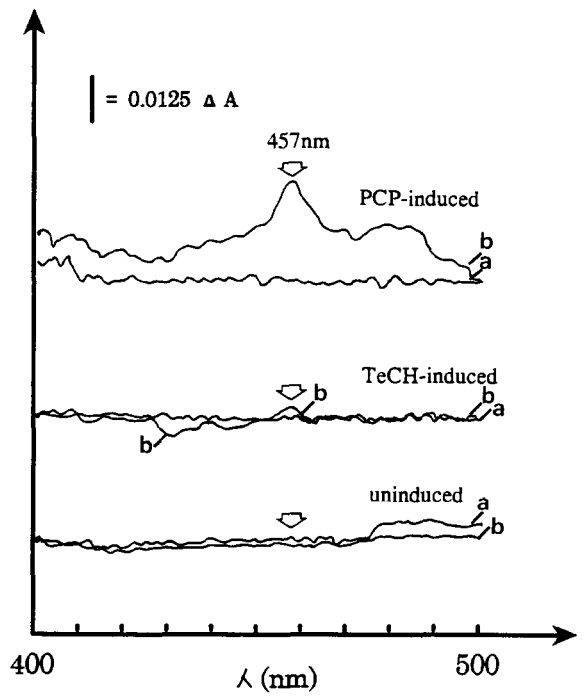

Fig. 2. Absorbance spectra (1 cm) of Triton X-100 (1% v/v)-dissolved membrane fraction (150000 × g, 8.4 mg protein ml^{-1}) prepared from PCP-, TeCH-, and uninduced cells of *R. chlorophenolicus* PCP-I. (a) membrane suspension reduced with sodium dithionite (10 mM), (b) as above, and then purged with CO.

Carbon monoxide (p_{co} = 0.1) decreased the activity by 70% in the presence of sodium dithionite, whereas the same amount of sodium dithionite alone had little effect (Table 3). Methylenebisthiocyanate, a chelator of ferrous iron, inhibited the activity, but a chelator of ferric iron (Tiron) did not.

The involvement of cytochrome P_{450}

To get direct evidence for the involvement of cytochrome P_{450} in the dechlorination, the carbon monoxide differential spectrum of the prereduced dissolved membrane fraction was measured. Figure 2 shows the spectra of the dithionite-reduced membrane fraction from PCP-, TeCH- and uninduced cells of *R. chlorophenolicus* PCP-I. An increase in the absorbance ($\Delta A_{1\,cm}$ = 0.0262) at 457 nm was seen when dissolved membranes from PCP-induced cells were reduced with dithionite and then purged with carbon monoxide. The peak at 457 nm in the differential spectrum with dithionite and carbon monoxide versus dithionite alone indicates the presence of a cytochrome P_{450}-type coenzyme. Little or no change in the absorbance by carbon monoxide ($\Delta A_{1\,cm} < 0.0025$, detection limit) was observed between 400 nm and 500 nm in the sodium dithionite reduced membrane fractions from TeCH-induced or uninduced cells (Fig. 2), indicating specific involvement of cytochrome P_{450} in the *para*-hydroxylation of PCP.

Discussion

Mono- and dichlorophenol-dechlorinating enzymes have been isolated, purified and the genes cloned. Removal of chlorine from benzene ring after the ring opening is also known in great detail (for a review, see Reineke & Knackmuss 1988). Two reports described the dechlorination of polychlorinated aromatic compounds in a microbial cell free system (Apajalahti & Salkinoja-Salonen (1987b; Schenk et al. 1989). Schenk et al. (1989) demonstrated the *para*-hydroxylation of PCP into TeCH by a membrane fraction of *Arthrobacter* sp. The reaction required both O_2 and NADPH for activity. Apajalahti & Salkinoja-Salonen (1987a, b) showed that *R. chlorophenolicus* PCP-I dechlorinated PCP into TeCH and further into a non-chlorinated ring-fission precursor 1,2,4-trihydroxybenzene.

The present paper demonstrates PCP *para*-hydroxylation in cell extract of PCP-induced *R. chlorophenolicus* PCP-I. The activity was located within the membrane fraction, in contrast to the TeCH-dechlorinating enzymes, which were soluble (Apajalahti & Salkinoja-Salonen 1987b; Uotila et al. 1990; and this paper). SKF-525A, metyrapone and menadione (cytochrome P_{450} inhibitors, for a review, see Ortiz de Montellano 1986) and the absence of O_2 inhibited PCP dechlorination in the cell-free system. Introduction of carbon monoxide (P_{co} = 0.1) into sodium dithionite-reduced membrane extract also blocked the PCP dechlorination and resulted in an increase of absorbance at 457 nm

when PCP-induced cells were used as a source membrane. Such a shift was not seen in membranes prepared from TeCH-induced and uninduced cells indicating that it was due to an enzyme specifically induced by PCP. The concentration of *cyt* P_{450} can be calculated from the spectral shift caused by carbon monooxide. Assuming an mM extinction coefficient for cytochrome P_{450} of $\Delta A_{450-490} = 92.8\,cm^{-1}$ (Gunsalus & Wagner 1978), there was 0.03 mmol of cytochrome P_{450} mg^{-1} of membrane protein in the PCP-induced cells and less than 0.003 nmol in the TeCH- or uninduced cells.

Cytochrome P_{450}-enzymes are broadly distributed among bacteria (for a review, see Asperger & Kleber 1990), but only one P_{450} enzyme with a membrane association has been described (*Acinetobacter calcoaceticus;* Müller et al. 1989).The data presented in this paper suggest that a cytochrome P_{450} type oxygenase may be involved in the membrane associated PCP *para*-hydroxylation of *R. chlorophenolicus* PCP-I. The requirement for the presence of molecular oxygen in PCP dechlorination was earlier shown within whole cells of *R. chlorophenolicus* PCP-I (Apajalahti & Salkinoja-Salonen 1987a), although we were unable to detect the incorporation of molecular oxygen into PCP. Many different cytochrome P_{450} enzymes require oxidized cofactors as a sink for reducing equivalents (Ortiz de Montellano 1986). Neither FAD nor $NADP^+$ relaxed the requirement for oxygen of the PCP *para*-hydroxylation in the cell free system of *R. chlorophenolicus* PCP-I. The true role of molecular oxygen in this reaction remains to be elucidated.

A cofactor requirement of the PCP dechlorination was observed when the membrane fraction was dissolved with detergent. Similar requirements have been reported for phenol and styrene hydroxylating enzymes (Liu & Chapman 1984; Straube 1987; Hartmans et al. 1990).

We also showed, that TeCH was O-methylated by the uninduced cell extracts. Interestingly, no such activity was observed in the extracts of PCP- and TeCH-induced cells. Constitutive O-methylation of chlorophenols has been demonstrated in several *Rhodococcus, Mycobacterium, Acinetobacter* and *Pseudomonas* species, with no involvement of dechlorination (Häggblom et al. 1988b; Suzuki 1978; Neilson et al. 1988).

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