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GLUTATHIONE IS THE REDUCING AGENT FOR THE REDUCTIVE DEHALOGENATION OF TETRACHLORO-*p*-HYDROQUINONE BY EXTRACTS FROM A *FLAVOBACTERIUM* SP.@

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Tetrachloro-*p*-hydroquinone is the first intermediate during pentachlorophenol degradation by *Flavobacterium* sp. strain ATCC 39723, a strict aerobe. We report here that tetrachlorohydroquinone was reductively dehalogenated to 2,3,6-trichloro-*p*-hydroquinone and subsequently to 2,6-dichloro-*p*-hydroquinone under anaerobic conditions by the cell extract from *Flavobacterium*. The reducing agent was identified to be the reduced form of glutathione. This is the first time glutathione has been identified as the reducing agent for reductive dehalogenation.

Reductive dehalogenation has long been recognized as one mechanism for the microbial dehalogenation of halogenated aromatic compounds (7). However, natural reducing agents required for reductive dehalogenases have yet to be identified. We have been working on the molecular biology of pentachlorophenol (PCP) degradation by *Flavobacterium* sp. strain ATCC 39723 and have reported the identification and purification of PCP hydroxylase which oxidizes PCP to tetrachlorohydroquinone (TeCH) in the presence of NADPH and O_2 (11,12). Whole cell studies with blocked mutants suggested that the *Flavobacterium* sp. reductively converts TeCH to 2,3,6-trichloro-*p*-hydroquinone (TrCH) and then to 2,6-dichloro-*p*-hydroquinone (DiCH), intermediates which are subsequently mineralized by as yet uncharacterized reactions (8). Here, we report the identification of the reductive dehalogenation of TeCH to TrCH and then to DiCH by the cell extract from the *Flavobacterium* and the identification of the reducing agent as the reduced form of glutathione (GSH).

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MATERIALS AND METHODS

Growth of cells and cell extract preparation. *Flavobacterium* sp. strain ATCC 39723 was cultured and PCP degradation was induced as previously reported (12). Cell extract was prepared by a French press essentially as described previously (12) except that the cell paste was suspended in 50 mM potassium phosphate (KPi) buffer at pH 7.4. For ammonium sulfate fractionation of proteins, ammonium sulfate was added to 20% saturation at room temperature (23°C). The precipitate was centrifuged (10,000 x g for 15 min) down and discarded. Additional ammonium sulfate was added to 60% saturation and the protein precipitate was collected by centrifugation (10,000 x g for 15 min).

Enzymatic activity assay. The reaction mixture contained 50 mM KPi buffer (pH 7.4), 0.4% Tween 20 (Sigma), 100 μM of TeCH, and 1 mM ascorbic acid. The concentrations of various reducing agents and protein used in the reactions are reported in the results. All the reactions were incubated at 23°C in an anaerobic chamber containing an atmosphere of 85% N_2, 5% CO_2, and 10% H_2 (Coy Laboratory Products Inc., Ann Harbor, Mich.). The reactions were stopped by addition of an equal volume of acetonitrile to the reaction mixture. TeCH stock solution (20 mM) was prepared in absolute ethanol. NADPH and NADH stock solutions (10 mM) were freshly prepared in 1 mM Tris buffer (pH 13) with 1 mM dithiothrietol. The stock solutions of FAD (10 mM), FMN (10 mM), and the reduced form of GSH (1 M) were freshly prepared in distilled water before use.

Analytical methods. DiCH was obtained from Aldrich Chemical Co. (Milwaukee, Wis). TrCH was made by enzymatic hydroxylation of 2,3,6-trichlorophenol by PCP hydroxylase (12). TeCH, TrCH and DiCH were analyzed by HPLC (Waters, Milford, Mass.) on a Nova-Pak C-18 column (3.9 by 150 mm) with an 11 mM H_3PO_4-acetonitrile gradient as described previously (12). Maximum absorption at 285, 300, 310 nm was recorded by using a Waters 490E programmable multiwave-length UV detector connected to a Maxima NEC computer workstation (Waters). The retention times of TeCH, TrCH and DiCH were 8.9, 8.1 and 6.7 min, respectively. TeCH and DiCH in the reaction mixtures were identified and quantified by comparison of their retention time and peak areas with that of authentic standards, respectively. TrCH was extracted with diethyl ether from the reaction mixture and analyzed by gas chromatography/mass spectrometry (GC/MS) as described previously (10). Since DiCH could not be extracted from the reaction mixture with ether, we collected the DiCH peak from the HPLC analysis using 0.1% trifluoroacetic acid instead of H_3PO_4 in the elution gradient. The collected DiCH was dried in a Speed-vac (Savant Instruments Inc. Farmingale, NY) and resuspended in a few microliters of ethyl acetate before GC/MS analysis (10). GSH and the oxidized form of glutathione (GS-SG) were detected by the enzymatic method of Tietze (9).

RESULTS

Detection of putative reductive dehalogenase activity. When TeCH was added to the cell extract of PCP-induced cells of *Flavobacterium* sp. strain ATCC 39723, TeCH concentration decreased, but TrCH and DiCH were not detectable by HPLC analysis (data not shown). Therefore, it was not clear if under these conditions, the disappearance of TeCH was due to chemical oxidation of TeCH or to enzymatic conversion. In order to address this question as well as protect the stability of the potential hydroquinones that would form from an enzymatic dehalogenation of TeCH, we incubated the reaction mixture in an anaerobic chamber. Under anaerobic conditions all three of the hydroquinones in question, that is TeCH, TrCH, and DiCH, would be stable.

Under these conditions, we detected both the decrease in the concentration of TeCH and the accumulation of both TrCH and DiCH. However, the dehalogenation activity was quite low, with half of the initial 100 μM TeCH being removed in 20 min. Incubation for an additional 24 h did not result in further metabolism of the remaining substrate.

Identification of the reducing agent. The conversion of TeCH to TrCH and DiCH by extracts suggested that TeCH metabolism was initiated by the removal of two chlorine atoms by reductive dehalogenation. Various compounds were tested as potential reducing agents. We tested NADPH, NADH, FAD, FMN, dithiothrietol, 2-mercaptoethanol, and the reduced form of glutathione (GSH) as potential reducing agents (Table 1). Only GSH stimulated the dehalogenation. The reaction was about 75 times faster than the control without the addition of GSH (Table 1). Although 1 mM ascorbic acid was added to reaction mixtures to prevent TeCH oxidation, it clearly was not a reducing agent for dehalogenation because the rate was the same when the ascorbic acid was replaced with 1 mM dithiothrietol.

Confirmation of enzymatic reaction. Cell extracts were prepared from both PCP-induced and uninduced cells of *Flavobacterium* sp. strain ATCC 29723. GSH-dependent reductive dehalogenation of TeCH was detected in both cell extracts from induced and uninduced cells at about the same level. The enzymatic activities of the cell extracts from both uninduced and PCP-induced cells were completely inactivated after treatment in a boiling water bath for 5 min.

Confirmation of the intermediate and end products. Tri- and dichlorohydroquinone are the intermediate and end product, respectively. TeCH was added to an anaerobic, GSH-supplemented reaction mixture containing extract from PCP-induced cells. Samples were taken at 5 min intervals and subjected to HPLC analysis. Two peaks accumulated sequentially, the

Table 1. Evaluation of cofactors for reductive dehalogenation

Cofactors (500 μM)	Dehalogenation rate +/- SD[a] μmol/min per g
Control	0.030 +/- 0.003
NADPH	0.027
NADH	0.028
FAD	0.026
FMN	0.028
Dithiothrietol	0.031
2-mercapto-ethanol	0.029
GSH	2.260 +/- 0.230

[a] Triplets of control (no cofactor addition) and sample with GSH were tested. Cell extracts were from PCP-induced *Flavobacterium* cultures. A single detection of the samples containing the other cofactors was reported. Protein concentration in the reaction mixture was 14.8 mg/ml except the sample with GSH which contained 7.4 mg/ml.

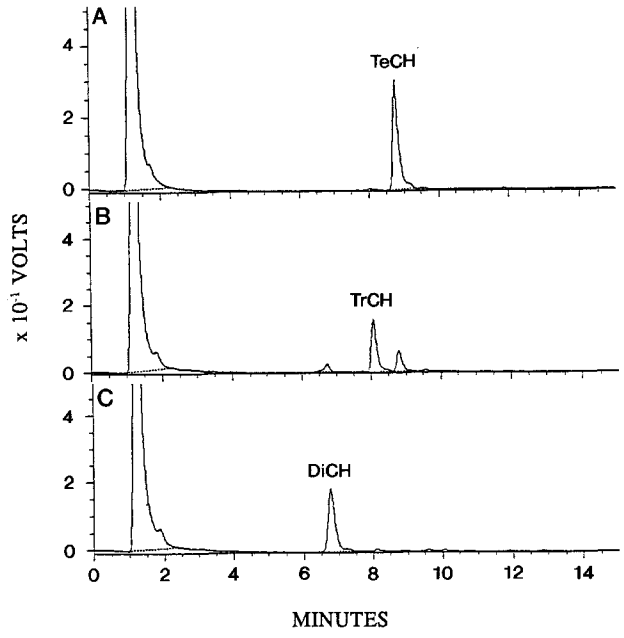

Fig. 1. Reductive dehalogenation of TeCH to TrCH and ultimately to DiCH by the cell extract from PCP-induced cultures. The reaction mixture contained 7.4 g protein/L and 1 mM GSH. The hydroquinones were monitored by a UV detector, and the relative absorbance was converted to volts by a computer. Figure 1A represents time zero of the reaction. Figure 1B was after the 5 min of incubation, and Figure 1C was after 10 min of incubation.

first to appear co-migrating with TrCH, and the second co-migrating with DiCH (Fig. 1). The HPLC peak that corresponded to DiCH was collected and subjected to analysis by GC/MS. A peak with a retention time corresponding to that of authentic DiCH (5.5 min) and a base peak at M/Z (M+) 178 corresponding to a molecular formula of $C_6H_4O_2Cl_2$ was found (data not shown). Major ions were observed at M/Z 180 (M+2), and 114. The retention time and major ions were the same as that for authentic TeCH.

TrCH, enzymatically generated from 2,3,6-trichlorophenol as specified in the Materials and Methods, displayed the same HPLC retention time as TrCH resulting from the reductive dehalogenation of TeCH by the cell free extract, and was converted to DiCH by the cell-free extract (data not shown).

GSH is oxidized during reductive dehalogenation. PCP-induced cell-free extract was fractionated by ammonium sulfate precipitation and the fractions tested for their ability to catalyze reductive dehalogenation. The fraction from 20 to 60% saturation at room temperature was found to be the most active. One gram of the ammonium sulfate-precipitated proteins was suspended in 1 ml of distilled water and dialyzed against 1 liter of 50 mM KPi buffer (pH 7.4)

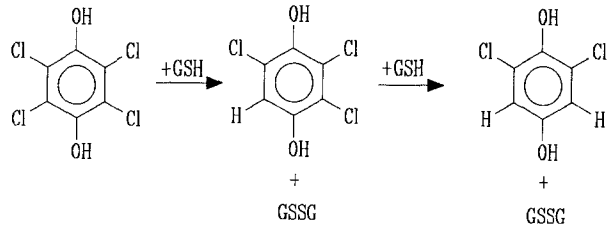

Fig. 2. Proposed reductive dehalogenation reaction by cell extracts from *Flavobacterium* sp. strain ATCC 39723.

at 6°C for 4 hours. The dialysate was assayed for reductive dehalogenation activity during a 1 h anaerobic incubation following the addition of 500 μM of NADPH, NADH, dithiothrietol, 2-mercaptoethanol, or GSH, or without any addition. Only 1 mM GSH restored the dehalogenase activity. The results suggest that there may be sufficient reduced GSH in the anaerobic cell free extract to support some dehalogenation and this GSH is removed by ammonium sulfate precipitation and dialysis.

The complete conversion of 100 μmoles of TeCH to DiCH by a cell-free extract was accompanied by the consumption of 229 μmol of GSH and the production of 108 μmol of oxidized glutathione (GS-SG). Furthermore, in a TeCH-free control, no GSH was oxidized indicating that the oxidation was dehalogenation-dependent. Theoretically, 400 μmol of GSH should be utilized for the conversion of 100 μmol of TeCH to DiCH. We believe that some GS-SG was reduced by the reducing environment in the anaerobic chamber during the 30 min incubation.

These results are consistent with the pathway of GSH-dependent metabolism proposed in Figure 2.

DISCUSSION

The reductive dehalogenation of TeCH to TrCH and DiCH in the cell extract from the aerobic *Rhodococcus chlorophenolicus* has been reported before (1,5). However, the authors did not determine the reducing agent for the reaction. Similarly, the reductive dehalogenation was also reported for 3-chlorobenzoate in the cell extract from the anaerobe, *Desulfomonile tiedjei* (2). The authors found that the cell extract utilized a dye, methyl viologen, as the reducing agent, and they were unable to identify a cellular compound as the reducing agent. Because methyl viologen has a very low redox potential (-424 mV), it theoretically can reduce GS-SG to 2GSH. Of course, we are not sure if GSH is the reducing agent for dechlorination of 3-chlorobenzoate because GSH was not tested in the report (2). The only reported reductive dehalogenase, a flavoprotein iodotyrosine deiodinase, was from bovine thyroid (4). The enzyme

was purified based on the dithiothrietol supported enzyme activity; however, the natural reducing agent was not identified.

GSH-dependent dehalogenase of dichloromethane and dibromomethane was purified from *Hyphomicrobium* (6). However, the GSH was not consumed during the dehalogenation, and GSH was simply proposed as a catalyst (6). In the enzymatic conversion of TeCH to TrCH and then to DiCH, GSH was oxidized to GS-SG. Therefore, GSH is a cosubstrate of the corresponding enzyme(s) and the reducing agent for the reaction. GSH should not be a limiting factor in *Flavobacterium*, as it is the major thiol-containing compound found in other facultative and aerobic gram-negative bacteria, such as *Escherichia coli* and *Pseudomonas fluorescens* (3). In summary, GSH is the only identified natural reducing agent for enzyme-mediated reductive dehalogenation.

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