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# INCORPORATION OF [$^{18}$O]WATER INTO 4-HYDROXYBENZOIC ACID IN THE REACTION OF 4-CHLOROBENZOATE DEHALOGENASE FROM PSEUDOMONAS SPEC. CBS 3

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Summary: 4-Chlorobenzoate is dehalogenated by 4-chlorobenzoate dehalogenase from Pseudomonas spec. CBS 3 to form 4-hydroxybenzoate. In $^{18}$O enriched water the hydroxygroup of 4-hydroxybenzoate is quantitatively labelled with $^{18}$O. This result clearly shows, that 4-chlorobenzoate dehalogenase catalyzes the hydrolytic cleavage of the halogen-carbon bond, without the involvement of molecular oxygen, a reaction not yet described. 

Introduction: The presence of a halogen substituent often prevents complete biodegradation of aromatic compounds (1). Sometimes the halogenated metabolites even prevent the degradation of the otherwise easily degraded unsubstituted compounds (2,3). The removal of halogen substituents in an early degradation step, however, could avoid such problems. Previously we reported the isolation of a bacterium, Pseudomonas spec. CBS 3, which is able to degrade halogenated compounds like 4-chlorobenzoate and 4-chlorophenylacetate (4-8). In the degradation of these substances the chlorine is already removed in the first step. In the case of 4-chlorophenylacetate we were able to demonstrate, that this step is performed by a two component enzyme system (9). Molecular oxygen, NADH and $Fe^{2+}$ are necessary for the reaction and 3,4-dihydroxyphenylacetate is the reaction product. In contrast in the degradation of 4-chlorobenzoate by the same

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strain 4-hydroxybenzoate is the first intermediate. In this paper we show, that the enzymatic conversion of 4-chlorobenzoate to 4-hydroxybenzoate proceeds via a hydrolytic cleavage of the carbon-chloride bond.

Materials and Methods: 4-chlorobenzoate was a gift from Bayer AG, Leverkusen, Germany, 4-hydroxybenzoate was purchased from Fluka AG, Buchs, Switzerland, $^{18}O$ $H_2O$ was from Ventron GmbH, Karlsruhe, Germany. 4-Chlorobenzoate dehalogenase was enriched from extracts of Ps. spec. CBS 3 by ammoniumsulfate precipitation and gelpermeation chromatography. Details of the purification procedure will be described elsewhere.

To examine, if the reaction proceeds under anaerobic conditions, we incubated 4,2 mg 4-chlorobenzoate dehalogenase under nitrogen in 50 mM phosphate buffer containing 6,3 µM $MnSO_4 \times H_2O$, 1,1 mM 4-chlorobenzoate and a few grains of dithionite. To check, whether the solution was anaerob, we recorded the spectra of the reaction mixture between 400 and 330 nm every 2 min. After 30 minutes the reaction was stopped by the addition of 6 N HCl and the amount of product formed was determined.

For the isotope labelling experiments we dissolved in 1 ml of 90 % $^{18}O$ enriched $H_2O$ 100mg freeze dried powder of the enzyme containing phosphate buffer pH 7 to yield a final concentration of 50 mM buffer and 55 mg/ml protein. To this solution we added 0,1 mg $MnSO_4 \times H_2O$ and 1 mg 4-chlorobenzoate and stirred this solution overnight at room temperature. The same assay was performed in unlabelled water as a control experiment. In order to check, whether there is an exchange of the ring OH-group under the conditions used we added in another assay 0,5 mg of 4-hydroxybenzoate instead of the 4-chlorobenzoate in labelled water. The assays were freeze dried and redissolved in 1 ml 1 N HCl. The obtained solutions were extracted five times with 2 ml ethylacetate. The ethylacetate was removed in vacuo and to the residues we added a solution of diazomethane in ether till the yellow color remained. The ether was removed, and the residues were dissolved in 50 µl of methanol. For GC-MS analysis 0,1 µl of this solution was injected onto a capillary gas chromatography column (SE30, 25 m, 120°C, injector 250°C), which was connected to a mass spectrometer (Varian 3700).

Results and Discussion:

The enzyme catalyzed substitution of a halogen at an aromatic ring by a hydroxygroup is a previously not described reaction. Usually the insertion of a hydroxygroup into aromatic compounds is catalyzed by oxygenases. In the case of the

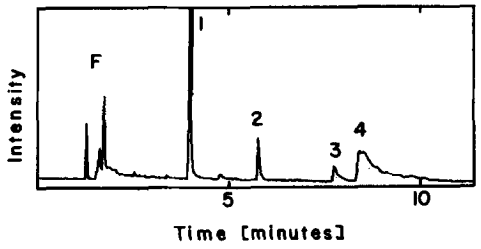


**Figure 1: Gas chromatogram of the reaction mixture containing 4-chlorobenzoate dehalogenase and 4-chlorobenzoate after methylation with diazomethane as described in methods. The peaks were : 1 = benzoic acid methylester, 2 = 4-hydroxybenzoic acid methylester, 3 = unknown compound, 4 = 4-methoxybenzoic acid methylester, F = solvent front.**

dehalogenation of 4-chlorobenzoate by 4-chlorobenzoate dehalogenase no oxidation is necessary, since the carbon 4 retains its valence in this reaction. Still the possibility remains, that the oxygen in the hydroxygroup is derived from molecular oxygen. To check this possibility we performed the reaction under strictly anaerobic conditions. In the anaerobic assay about 80 % of the amount of product of the aerobic control assay were formed, indicating, that molecular oxygen was not necessary for the reaction, and that the oxygen in the product is derived from water. To ensure this, we performed the reaction in 90 % $^{18}O$ labelled water. Figure 1 shows a typical chromatogramm of the reaction mixture obtained after methylation as described in methods. Besides the methylated substrate two major peaks were obtained, one corresponding to 4-hydroxybenzoaic acid methylester, the other one belonging to 4-methoxybenzoic acid methylester. Figure 2 shows the mass spectra of the twofold methylated compound, when the reactions were performed in labelled and in unlabelled water. From the peaks at m/e = 109/107 ($M^+$-$COOCH_3$) and at m/e = 94/92 ($M^+$-$COOCH_3$, -$CH_3$) we calculated, that 82 % of the ring hydroxygroup have been labelled. In the control, where 4-hydroxybenzoate was incubated with the

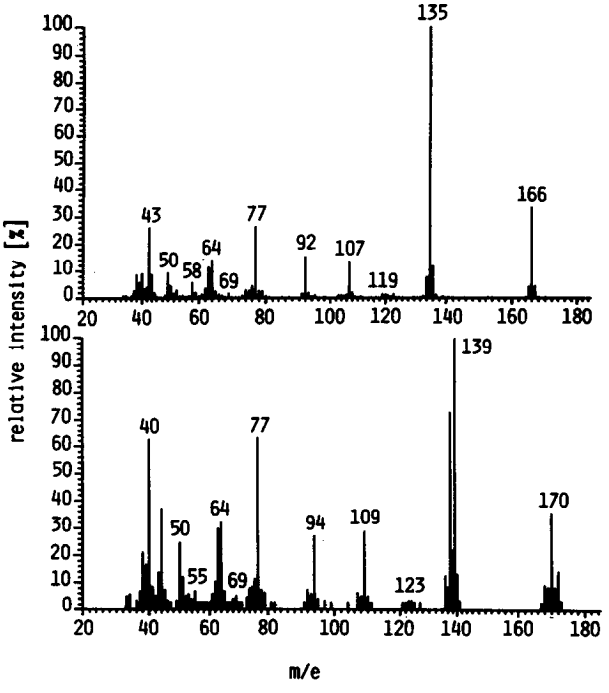


**Figure 2: Mass spectra of 4-methoxybenzoic acid methylester obtained after methylation of the reaction product of 4-chlorobenzoate dehalogenase, when the reaction was performed in unlabelled (upper) and in 90 % $^{18}O$ labelled water (lower).**

enzyme in labelled water no label was found in this hydroxy-group. These data clearly show, that the oxygen in the enzymatically formed 4-hydroxybenzoate is derived from water. We therefore conclude, that the removal of the chlorine from 4-chlorobenzoate by 4-chlorobenzoate dehalogenase is a hydrolytic cleavage of the carbon-halogen bond. Such a hydrolytic cleavage has been reported in the degradation of several chlorinated aliphatic compounds (10-13).In the degradation of chlorinated aromatic compounds, however, usually the chlorine is removed after cleavage of the aromatic ring (for review see ref. 14, 15). In the case of 4-chlorobenzoate the removal of halogen in the initial step has been described in several microorganisms (4,5,16,17). Unfortunately none of the enzymes catalyzing this interesting reaction has been purified.

Zaitsev and Karasevich (17) proposed from manometric experiments with whole cells of Arthrobacter globiformis, that the hydroxygroup of 4-hydroxybenzoate is derived from water. With the above described experiments this suggestion is confirmed enzymatically.

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