Resolution of 4-Chlorobenzoate Dehalogenase from Pseudomonas sp. Strain CBS3 into Three Components†

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Extracts of Pseudomonas sp. strain CBS3 grown with 4-chlorobenzoate as sole carbon source contained an enzyme that converted 4-chlorobenzoate to 4-hydroxybenzoate. This enzyme was shown to consist of three components, all necessary for the reaction. Component I, which had a molecular weight of about 3,000, was highly unstable. Components II and III were stable proteins with molecular weights of about 86,000 and 92,000.

Due to their widespread use and their recalcitrance, many chlorinated compounds are found as primary pollutants in our environment. Nevertheless, several bacteria have been isolated which possess the ability to degrade chlorinated hydrocarbons (8). Of special interest in these bacteria are the enzymes that catalyze the release of the halogen substituent. Several bacteria with the ability to use 4-chlorobenzoate as sole carbon source have been isolated (1, 3-5, 11, 13, 14). All of these bacteria degraded 4-chlorobenzoate via 4-hydroxybenzoate, which implies that the initial degradation step is a dehalogenation. However, in only a few cases could the corresponding enzyme activities be detected (5, 9, 12). It was demonstrated in two cases that the newly introduced hydroxy group was derived from water and that molecular oxygen was not necessary for the reaction (6, 10). These enzymes are the only examples in which a halogen at the aromatic ring is substituted by a hydroxy group without the involvement of molecular oxygen. It would be interesting to investigate the mechanism of this unusual reaction. A prerequisite for such investigations is a pure preparation of such an enzyme. Despite many efforts, to date none of these enzymes has been purified to homogeneity. The loss of most or all of the activity in all purification procedures, as experienced by several groups (12), was mainly attributed to the instability of the enzymes. Here we present evidence that the enzyme from Pseudomonas sp. strain CBS3 is a multi-component system and that the separation of the components is one of the reasons for this loss in activity.

In crude extracts prepared by ultrasonic treatment of Pseudomonas sp. strain CBS3 an enzyme was detectable which hydrolytically converted 4-chlorobenzoate to 4-hydroxybenzoate (10, 12). When we fractionated these extracts by ammonium sulfate precipitation, in the fraction between 0 and 40% saturation only about 5% of the activity was detectable. In the fraction between 40 and 60% saturation up to 20% of the activity was recovered. However, fractionation between 0 and 60% saturation resulted in up to 90% of the activity. When the fractions from 0 to 40% and 40 to 60% saturations were combined, up to 80% of the activity was restored. These results strongly suggested that 4-chlorobenzoate dehalogenase included at least two components which were separated by ammonium sulfate precipitation. The component present in the 40 to 60% precipitate, termed component I, could not be resolved further. The precipitate between 0 and 40% ammonium sulfate saturation in 20 mM potassium phosphate buffer was applied to a hydroxyapatite column. When the fractions obtained from the hydroxyapatite column were tested in the presence of component I for dehalogenation of 4-chlorobenzoate, no activity was detectable. However, when the fractions that were eluted with the phosphate gradient were mixed with component I and with the protein fraction that had passed the column unretarded, dehalogenating activity was present in the fractions which were eluted at around 120 mM phosphate (Fig. 1). These results suggested that the precipitate between 0 and 40% ammonium sulfate saturation contained at least two more components necessary for the dehalogenation reaction. The component in the flowthrough was termed component II, and the component eluted with phosphate was denoted component III.

Unfortunately, component I was highly unstable. At 4°C, within 24 h >50% of the activity was lost. In gel filtration with Sephacryl S200, the component migrated at the lower exclusion limit, indicating a molecular weight below 10,000. On Sephadex G25 it migrated close to the front, suggesting a molecular weight above 1,000. On Sephadex G50 the activity was detectable at a molecular weight of about 3,000 ± 2,000. However, in all cases no more than 5% of the activity applied to the column was detected after the column. Due to the low activity recovered from the column, several fractions had to be combined to detect any activity, which led to the inaccuracy in the molecular weight determinations. Component I could not be replaced by any of the well-known cofactors such as NAD, NADH, NADP, NADPH, flavin adenine dinucleotide, reduced flavine adenine dinucleotide, ATP, ADP, AMP, acetyl-coenzyme A, malonyl-coenzyme A, and divalent metal ions such as Mn2+, Co2+, Zn2+, and Fe2+, and these cofactors had no effect on the stability of

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FIG. 1. Separation of components II and III of 4-chlorobenzoate dehalogenase from Pseudomonas sp. strain CBS3 on a hydroxyapatite column.

component I. Components II and III were perfectly stable proteins. In gel permeation chromatography on Sephadex G150 and Sephacryl S300, the molecular weights of the two components were determined to be about 86,000 and 92,000, respectively.

The activity of each component in fresh crude extracts was determined as described previously (12) after the addition of an excess of the other two components. As expected, the labile component I was limiting for the overall activity in crude extracts from Pseudomonas sp. strain CBS3 (1.26 mU/mg of protein). It is interesting to note that the activity of component III (19.8 mU/mg of protein) was about sixfold higher than that of component II (3.5 mU/mg of protein).

The results described show that for the dehalogenation of 4-chlorobenzoate by Pseudomonas sp. strain CBS3 at least three different components were necessary. Only when all three components were present was the activity detectable. This might explain why all attempts to purify the dehalogenase so far had failed. In all purification steps at least one of the components was separated from the others, leaving the enzyme inactive. In contrast, the loss of activity in crude extracts was clearly due to the instability of the small component I. So far we have not been able to stabilize this compound, and due to its instability it is difficult to determine its chemical nature. Its low molecular weight suggests that it may be a common cofactor. However, none of the cofactors we have tested so far has been able to substitute for component I. Certainly the disclosure of its nature would help to understand the mechanism of this unusual reaction.

Several multicomponent enzyme systems have been described that dehalogenate chlorinated aromatic compounds, e.g., 4-chlorophenylacetate dioxygenase (7) and 2-chlorobenzoate dioxygenase (2). In these enzyme systems the functions of the various components are well studied. The terminal oxygenase reacts with the substrate and oxygen to form the reaction product, whereas the other components, the reductases, deliver the electrons from NADH, which are necessary for the reaction. In the case of 4-chlorobenzoate dehalogenase, however, the situation is different. This enzyme requires neither oxygen nor NADH. Therefore, the function in a redox chain for the different components seems highly unlikely. To understand the mechanism of dehalogenation, purification and further characterization of all three components, now in progress in our laboratory, arc necessary.

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