Enzymic Dehalogenation of 4-Chlorobenzoyl Coenzyme A in *Acinetobacter* sp. Strain 4-CB1

SHELLEY D. COPLEY* and GWEN P. CROOKS

Cooperative Institute for Research in Environmental Science and Department of Chemistry and Biochemistry, *University of Colorado at Boulder, Boulder, Colorado 80309-0215

Received 25 October 1991/Accepted 13 January 1992

4-Chlorobenzoate degradation in cell extracts of *Acinetobacter* sp. strain 4-CB1 occurs by initial synthesis of 4-chlorobenzoyl coenzyme A (4-chlorobenzoyl CoA) from 4-chlorobenzoate, CoA, and ATP. 4-Chlorobenzoyl CoA is dehalogenated to 4-hydroxybenzoyl CoA. Following the dehalogenation reaction, 4-hydroxybenzoyl CoA is hydrolyzed to 4-hydroxybenzoate and CoA. Possible roles for the CoA moiety in the dehalogenation reaction are discussed.

Complete degradation of 4-chlorobenzoate can be accomplished by a number of microorganisms (1, 4, 7, 12, 16, 18). The pathway originally proposed for 4-chlorobenzoate degradation is shown in Fig. 1 (14). The initial step of the pathway was believed to be a hydrolytic dehalogenation of 4-chlorobenzoate because the reaction proceeds in the absence of oxygen (1, 7, 12, 16). Furthermore, *18O* labeling experiments have shown that the oxygen atom in the hydroxyl group of the product is derived from H$_2$O (8, 13). Recent reports indicate that 4-chlorobenzoate dehalogenation in extracts of *Pseudomonas* sp. strain CBS3 requires ATP and coenzyme A (CoA) and proceeds via initial formation of 4-chlorobenzoyl CoA (6, 15). Below we describe evidence that degradation of 4-chlorobenzoate in *Acinetobacter* sp. strain 4-CB1 also occurs through the intermediacy of 4-chlorobenzoyl CoA and that the actual substrate for the dehalogenase is 4-chlorobenzoyl CoA.

*Acinetobacter* sp. strain 4-CB1 was grown at 30°C in minimal salts medium (1) supplemented with trace element solution (0.5 ml/liter) (5), vitamin solution (0.5 ml/liter) (17), and 4-chlorobenzoate (0.4 g/liter). Cells were harvested by centrifugation and washed with cold phosphate buffer (100 mM, pH 7.0) containing 15% sucrose. The pellet was resuspended in phosphate buffer (100 mM, pH 7.0) containing phenylmethanesulfonyl fluoride (2 mM). Cells were disrupted by two passes through a French press at 20,000 lb/in$^2$. The lysate was centrifuged at 27,000 $\times$ g for 25 min at 4°C.

Dehalogenase assays were carried out by mixing extracts with either 4-chlorobenzoyl CoA (0.1 mM) or 4-chlorobenzoate (1 or 0.2 mM), CoA (0.1 mM), ATP (1 mM), MgSO$_4$ (1 or 5 mM) and (in some cases) an ATP-regenerating system consisting of phosphocreatine (10 mM) and creatine phosphokinase (0.1 mg/ml). The mixture contained either phosphate buffer (25 mM, pH 7.2) or Tris-HCl (25 mM, pH 7.2). Aliquots were removed at intervals for high-performance liquid chromatography (HPLC) or for phosphate determination. HPLC analysis was carried out by using a C-18 column with mobile phases consisting of mixtures of sodium acetate buffer (50 mM, pH 5.2) and methanol. Phosphate was assayed as described by Ames (2).

Dehalogenation of 4-chlorobenzoate by extracts of *Acinetobacter* sp. strain 4-CB1 is stimulated by the combination of ATP and CoA (Fig. 2), as previously observed for extracts from *Pseudomonas* sp. strain CBS3 (6, 15). Degradation of 4-chlorobenzoate was coupled to the hydrolysis of ATP (data not shown). Stimulation of the rate of phosphate release from ATP in the presence of 4-chlorobenzoate occurred only when both ATP and CoA were present. These data suggest that degradation of 4-chlorobenzoate may proceed via intermediate formation of the CoA thioester.

The CoA thioester of 4-chlorobenzoate was prepared to provide a standard for comparison with compounds observed during 4-chlorobenzoate degradation. 4-Chlorobenzoyl CoA was synthesized from CoA and 4-chlorobenzoyl chloride in KHCO$_3$ buffer (0.2 M). The CoA concentration was monitored (3), and when no CoA remained, the solution was recovered, leaving behind undissolved 4-chlorobenzoyl chloride. 4-Chlorobenzoyl CoA was purified by anion-exchange chromatography with a gradient of triethylammonium bicarbonate (20 to 800 mM, pH 7.5). Triethylammonium bicarbonate was removed by rotary evaporation from isopropanol.

In order to determine whether 4-chlorobenzoyl CoA is formed during degradation of 4-chlorobenzoate in *Acinetobacter* extracts, cell extracts were mixed with 4-chlorobenzoate, ATP, CoA, and Mg$^{2+}$ and the progress of the reaction was followed by HPLC. Accumulation and disappearance of 4-chlorobenzoyl CoA was observed (Fig. 3).

The fate of 4-chlorobenzoyl CoA in *Acinetobacter* cell extracts was determined by mixing 4-chlorobenzoyl CoA with cell extracts and following the progress of the reaction by HPLC. Figure 4 shows the disappearance of 4-chlorobenzoyl CoA, the formation of an intermediate, and the eventual formation of CoA and 4-hydroxybenzoate. The UV-visible spectra of the intermediate (collected from the HPLC column) at neutral and alkaline pH resemble those of 4-hydroxybenzoyl CoA published by Webster et al. (19) (data not shown). Furthermore, the intermediate coelutes during
HPLC with authentic 4-hydroxybenzoyl CoA synthesized as described by Merkel et al. (9) and purified by anion-exchange chromatography. (The identity of the synthetic 4-hydroxybenzoyl CoA was confirmed by comparison of its $^1$H-nuclear magnetic resonance spectrum with that published by Mieyal et al. [10].)

The data presented above demonstrate that 4-chlorobenzoyl CoA is produced and dehalogenated during the degradation of 4-chlorobenzoate in Acinetobacter extracts. On the basis of our results, the pathway for 4-chlorobenzoate degradation in Acinetobacter sp. should be amended as shown in (Fig. 5). A similar pathway has been demonstrated for 4-chlorobenzoate degradation in Pseudomonas sp. strain CBS3 (6, 15).

The actual substrate for the dehalogenation reaction during degradation of 4-chlorobenzoate by Acinetobacter sp. strain 4-CB1 is 4-chlorobenzoyl CoA. The reason for the thioesterification of 4-chlorobenzoate is unknown, although three possible roles for the CoA moiety may be proposed. One possible role of CoA is to retain hydrophobic metabolites in the cytoplasm by preventing diffusion through the cellular membrane. A second possible role of CoA is to serve as a "handle" by providing a large number of binding sites for recognition by enzymes. In addition to optimizing recognition, the binding energy thus achieved might be utilized in catalyzing the chemical transformation. A third possible role of CoA is to actually facilitate the dehalogenation reaction itself. A thioester is a more electron-withdrawing substituent than a carboxylate. Thus, the CoA might stabilize a negatively charged transition state and accelerate the dehalogenation reaction. However, the importance of the CoA in facilitating the dehalogenation reaction depends upon the mechanism of the enzymic reaction.

One possible mechanism for the dehalogenation reaction is the $S_n$Ar mechanism shown in Fig. 6. The dependence of the rate of $S_n$Ar reactions in substituted chlorobenzenes upon the nature of the substituents has been studied by Miller and Kai-Yan (11), who demonstrated a linear relationship between $\log k$ and the electron-withdrawing ability of the substituent (quantitatively expressed as a $\sigma$ value).

From their data, we estimate that the rate of substitution of 4-chlorobenzooyl CoA would be approximately $1.5 \times 10^6$-fold.
faster than that of 4-chlorobenzoate in a nonenzymic reaction. Therefore, if the reaction catalyzed by 4-chlorobenzoyl dehalogenase proceeds by the S_NAr mechanism, the presence of the CoA moiety would be a powerful accelerating force. However, the effect of the CoA thioester might be less dramatic if the dehalogenase reaction proceeds via some other mechanism, such as the benzoyl mechanism, the S_RN1 mechanism or the S_DN2 mechanism. Indeed, the rate of an S_DN2 reaction would actually be expected to be lower for 4-chlorobenzoyl CoA than for 4-chlorobenzoate because the electron-withdrawing thioester substituent would destabilize the positively charged intermediate. Our future work will be directed at differentiating between the possible reaction mechanisms and, in particular, discovering the means by which the enzyme catalyzes this unusual dehalogenation reaction.

We are grateful to Peter Adriaens and Dennis Focht for a sample of Acinetobacter sp. strain 4-CB1 and to Robert Kuchta and Tad Koch for helpful discussions.

REFERENCES