Volume 290, number 1,2, 224–226 FEBS 10212 September 1991

ADONIS 001457939100923E

Identification of 4-chlorobenzoyl-coenzyme A as intermediate in the dehalogenation catalyzed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3

Frank Löffler and Rudolf Müller

Technische Universität Hamburg-Harburg, Arbeitsbereich Biotechnologie II, POB 901052, 2100 Hamburg 90, Germany

Received 17 July 1991

The intermediate in the reaction catalyzed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3 was identified as 4-chlorobenzoyl-CoA. One component of 4-chlorobenzoate dehalogenase worked as a a 4-chlorobenzoyl-CoA ligase catalyzing the formation of 4-chlorobenzoyl-CoA from 4-chlorobenzoate, coenzyme A and ATP. This intermediate was detected spectrophotometrically and by HPLC. 4-chlorobenzoyl-CoA was the substrate for the dehalogenase component, which catalyzed the conversion to 4-hydroxybenzoate with concomitant release of coenzyme A.

4-Chlorobenzoate; 4-Chlorobenzoate dehalogenase; 4-Chlorobenzoyl-CoA; Dehalogenation; Degradation of chlorinated hydrocarbons; *Pseudomonas* sp. CBS3

1. INTRODUCTION

It is known that many microorganisms, especially bacteria, mineralize a multitude of halogenated hydrocarbons, at least under laboratory conditions. Despite the chemical stability of many halogenated hydrocarbons, bacterial enzymes are able to catalyze the release of the halogen substituents. It is therefore of great interest to elucidate the mechanisms of how these enzymes work. The 4-chlorobenzoate dehalogenase from the 4-chlorobenzoate (4-CBA) degrading bacterium *Pseudomonas* sp. CBS3 catalyzes the conversion of 4-CBA to 4-hydroxybenzoate (4-HBA) [1]. This dehalogenase was shown to be a two-component enzyme system [2]. The dehalogenating reaction prodeeded without the involvement of molecular oxygen and the newly introduced hydroxy group was derived from water [3]. Coenzyme A (CoA), ATP and Mg^{2+} were essential cofactors for the reaction. We have proposed a mechnism for the dehalogenating reaction as shown in Fig. 1 [4]. In this paper we describe the isolation and identification of 4-chlorobenzoyl-CoA as an intermediate in the dehalogenation reaction.

Dedicated to Prof. Dr. F. Lingens on the occasion of his 66th birthday.

Abbreviations: 4-CBA, 4-chlorobenzoate; 4-HBA, 4-hydroxybenzoate; CoA, coenzyme A.

Correspondence address: R. Müller, Technische Universität Hamburg-Harburg, Arbeitsbereich Biotechnologie II, POB 901052, 2100 Hamburg 90, Germany.

2. MATERIALS AND METHODS

2.1. *Organism and growth conditions*

The isolation, characterization and growth conditions of *Pseudomonas* sp. CBS3 as well as the prepation of crude extracts and the separation of both components have already been described [1,2,5].

2.2. *HPLC equipment and chromatography procedure*

A high performance liquid pump (Pharmacia LKB HPLC Pump 2248) equipped with an auto-sampling injector (Gilson model 231 with dilutor M401), a photodiode array detector (waters 994) and a reversed phase column (LiChrospher 100 RP-18 (5 μm), 125 × 4 mm inner diameter, Merck) were used. The mobile phase was 50 mM phosphate, pH 5.5/2-propanol (90:10, v/v). The flow rate was 1.5 ml/min and detection was performed at 245 nm. All measurements were carried out at room temperature.

2.3. *Enzyme assays*

2.3.1. *4-CBA-CoA ligase*

The activation of 4-CBA to 4-chlorobenzoyl-CoA was followed by HPLC. The reaction mixture (400 μl) contained 100 mM potassium phosphate buffer, pH 7.0, 9 mM ATP, 1.25 mM CoA, 5 mM $MgCl_2$, 1.25 mM 4-CBA, and the dehalogenase component. The assay mixture was allowed to equilibrate at 25°C and then the reaction was started by the addition of substrate. The enzymatic conversion was terminated through the adjustment of the pH to 2, with 6 N HCl.

2.3.2. *4-CBA-CoA dehalogenase*

The substrate for the dehalogenase component, 4-chlorobenzoyl-CoA, was produced enzymatically with the ligase component. The reaction mixture (400 μl) contained 100 mM potassium phosphate buffer, pH 7.0, 4-chlorobenzoyl-CoA and the dehalogenase component. The enzymatic reaction was performed at 25°C and stopped by the addition of HCl.

3. RESULTS

When 4-CBA was incubated with CoA, ATP, Mg^{2+} and the purified ligase component of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3, a new peak

Published by Elsevier Science Publishers B.V.

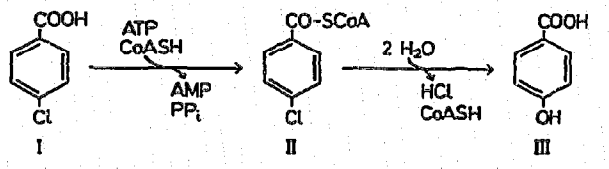

Fig. 1. The proposed mechanism for the reaction catalyzed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3.

with a retention time of 9 min was detected in HPLC. (peak II, Fig. 2c–2e). Under the chosen chromatography conditions ATP and CoA were eluted near the front. 4-CBA had a retention time of about 5 min (peak I, Fig. 2a). The decrease of the peak area of 4-CBA was correlated with the increase of the peak area of the reaction product, Without the addition of the ligase no additional peak was detected (Fig. 2a). Equally, no additional peak was detected when CoA was missing in the test system (Fig. 2b). Samples of peak II were collected and lyophilized. The residues were dissolved in 50 mM phosphate buffer, pH 7.0, and re-chromatographed. As expected, only a single peak with a retention time of 9 min was detected (peak II, Fig. 4a). Coenzyme A thioesters are known to be stable at acid pH but are rapidly hydrolyzed at alkaline pH values [6]. The obtained residues from peak II were dissolved in 50 mM phosphate buffer, pH 7.0, and spectra were recorded. The enzymatically formed substance revealed an absorbance maximum at 258 nm (Fig. 3, t=0). The spectrum remained unchanged at pH 2.0. After the adjustment of the pH with 10 N NaOH to pH 11 spectra were recorded after different times. Figure 3 shows the change of the spectra in dependence of the incubation time. During the incubation at alkaline pH the original spectrum with one maximum at 258 nm was transformed into a spectrum with two maxima at 237 and 257 nm in accordance with that of an equimolar mixture of 4-CBA and reduced CoA.

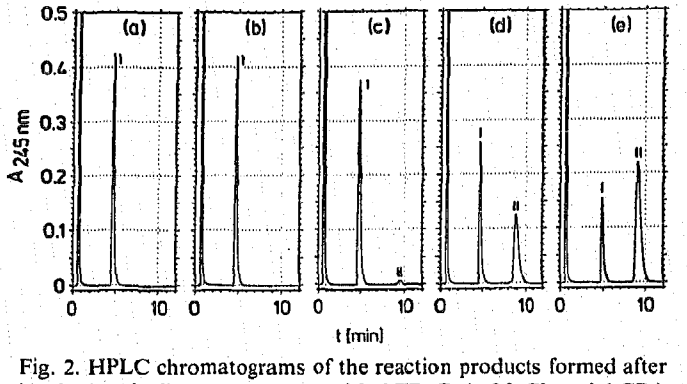

Fig. 2. HPLC chromatograms of the reaction products formed after incubating the ligase component with ATP, CoA, $MgCl_2$ and 4-CBA for 1 min (c), 40 min (d) and 100 min (e). No formation of the reaction product was observed when the ligase component (a) or CoA (b) were missing from the reaction mixture. I, 4-CBA; II, 4-chlorobenzoyl-CoA.

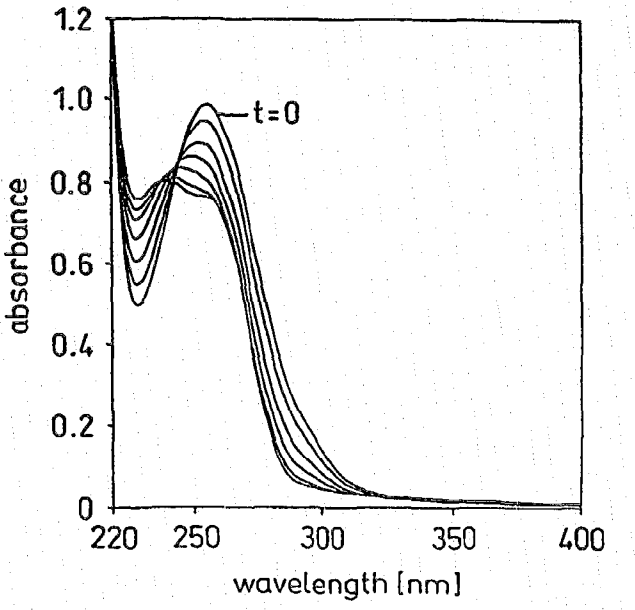

Fig. 3. Change of the UV-spectrum of enzymatically formed 4-chlorobenzoyl-CoA after the adjustment of the pH to 11. The spectra were recorded after 0.5, 15, 30, 60, 120 and 240 min at 10°C.

In another experiment the residues from peak II, obtained after purification by HPLC and lyophilization, were dissolved in 50 mM phosphate buffer, pH 7.0, adjusted to pH 11 with 10 N KOH and heated to 50°C for 15 min in a water bath. After this procedure a sample was subjected to HPLC. Peak II had disappeared. One peak near the front and one peak with a retention time idential to that of 4-CBA were detected (Fig. 4b). The spectrum of the peak at 5 min was identical with that of authentic 4-CBA. The peak at the front was identified as CoA.

After incubation of the reaction product of the above described reaction (peak II) with the dehalogenase component of 4-chlorobenzoate dehalogenase of *Pseudomonas* sp. CBS3, peak II disappeared. The disappea-

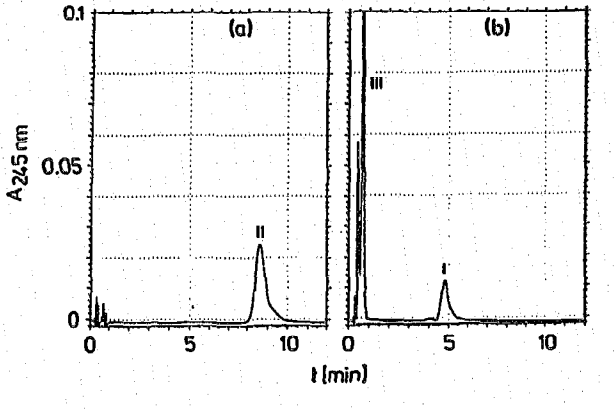

Fig. 4. HPLC chromatograms of the reaction products formed in the ligase reaction before (a) and after (b) incubation at alkaline pH. 4-chlorobenzoyl-CoA is hydrolyzed to 4-CBA and CoA. I, 4-CBA; II, 4-chlorobenzoyl-CoA; III, CoA.

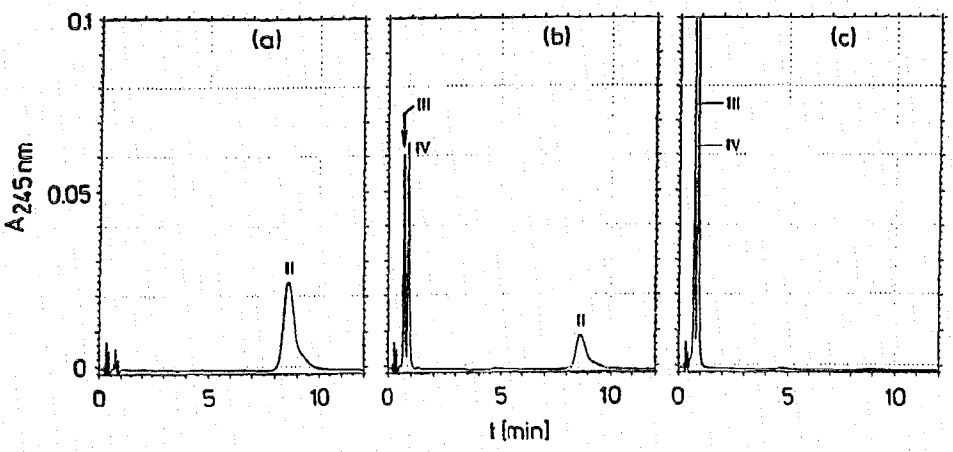

Fig. 5. Enzymatic of the reaction product of the ligase component without the dehalogenase component (a), with the dehalogenase component after 30 min (b), and after 120 min (c). The disappearance of peak II is coupled with the formation of CoA and 4-HBA. II, 4-chlorobenzoyl-CoA; III, CoA; IV, 4-HBA.

rance was associated with the development of 2 peaks near the front. These 2 peaks were identified on the basis of their spectra as CoA and 4-HBA, respectively (peak III and IV, Fig. 5b,c). After an incubation time of 120 min, peak II was completely converted to CoA and 4-HBA. Without the addition of the dehalogenase component no formation of CoA and 4-HBA was observed and peak II remained unaltered. From these results we concluded that peak II consisted of 4-chlorobenzoyl-CoA and was an intermediate in the dehalogenating reaction catalyzed by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3.

4. DISCUSSION

The dehalogenation of 4-CBA was an ATP and CoA dependent reaction, catalyzed by the 2 components of 4-chlorobenzoate dehalogenase. By analogy with other known ATP and CoA dependent enzymes, we have postulated a reaction sequence with 4-chlorobenzoyl-CoA as an intermediate [4]. The results described above substantiate this assumption. One component converted 4-CBA in the presence of ATP, CoA and Mg^{2+} to 4-chlorobenzoyl-CoA indicating that this component worked as a 4-chlorobenzoyl-CoA ligase. 4-Chlorobenzoyl-adenylate might be an enzyme-bound intermediate in this reaction [7]. 4-Chlorobenzoyl-CoA was the substrate for the other component. No cofactors were required fot the final dehalogenating reaction. The chlorine was substituted by an OH^- and CoA was split off. Water was utilized as the hydroxyl donor as shown by Müller et al. [3] in labelling experiments with $H_2{}^{18}O$. Hence, this component worked as a hydrolase. In accordance with the intermediate formation of a CoA-ester (see Fig. 1), in these labelling experiments in addition to the ring hydroxy group, one oxygen atom of the carboxy group was labelled. In contrast, Marks et al. [8], who worked with an *Arthrobacter* species, reported that only the ring hydroxy group of 4-HBA contained labelled oxygen. These data suggest that 4-chlorobenzoyl-CoA is not an intermediate in the dehalogenation of 4-CBA by *Arthrobacter*. This might indicate the existence of at least 2 different dehalogenating mechanisms for 4-CBA.

Acknowledgements: This work was supported by the Bundesministerium für Forschung und Technologie under the contract 0319416A and by the Fonds der Chemischen Industrie.

REFERENCES

[1] Thiele, J., Müller, R. and Lingens, F. (1987) FEMS Microbiol. Lett. 41, 115–119.
[2] Elsner, A., Löffler, F., Miyashita, K., Müller, R. and Lingens, F. (1991) Appl. Environ. Microbiol. 57, 324–326.
[3] Müller, R. Thiele, J., Klages, U. and Lingens, F. (1984) Biochem. Biophys. Res. Commun. 124, 178–182.
[4] Löffler, F., Müller, R. and Lingens, F. (1991) Biochem. Biophys. Res. Commun. 176, 1106–1111.
[5] Klages, U. and Lingens, F. (1980) Zbl. Bakt. Hyg. I. Abt. Orig. C1, 215–223.
[6] Webster, Jr. L.T., Mieyal, J.J. and Siddiqui, U.A. (1974) J. Biol. Chem. 249, 2641–2645.
[7] Londesborough, J.C. and Webster Jr, L. T. (1974) in: The Enzymes (Boyer, P.D. ed.) vol. 10, 3rd edn., pp. 469–488, Academic Press, New York.
[8] Marks, T.S., Wait, R., Smith, A.R.W. and Quirk, A.V. (1984) Biochem. Biophys. Res. Commun. 124, 669–674.