Separate cloning and expression analysis of two protein components of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. C8S3

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The two protein components, II and III, of the 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3 were cloned separately into *Escherichia coli*. Component II was obtained on plasmid pCBSII, containing a 3.0 kbp *HindIII* fragment, and component III on plasmid pCBSIIIb, containing a 1.3 kbp *SalI/PstI* fragment. The identities of the two components were confirmed by comparison with the authentic components from *Pseudomonas* sp. CBS3. Both components were expressed constitutively in *E. coli*. Neither component alone showed dehalogenating activity. Only in the mixture of crude extracts from both clones was 4-chlorobenzoate dehalogenase detectable. The specific activities in *E. coli* crude extracts were 2.9 mU (mg protein)⁻¹ for component II and 3.5 mU (mg protein)⁻¹ for component III. Expression analysis by minicell experiments revealed a single polypeptide chain of 29 kDa for component II and of 31 kDa for component III.

Introduction

Chlorinated benzoic acids are intermediates in the biodegradation of chlorinated biphenyls (Furukawa *et al.*, 1978) and herbicides (Köcher *et al.*, 1976). Several bacteria with the ability to utilize 4-chlorobenzoate as sole source of carbon and energy have been isolated (Ruisinger *et al.*, 1976; Klages & Lingens, 1979, 1980; Zaitsev & Karasevich, 1981; van den Tweel *et al.*, 1986; Marks *et al.*, 1984a). The initial step in 4-chlorobenzoate catabolism by these bacteria is the replacement of the chlorine substituent by a hydroxy group. Müller *et al.* (1984) and Marks *et al.* (1984b) showed that the oxygen in the enzymically formed 4-hydroxybenzoate was derived from water and that molecular oxygen was not necessary for the reaction. The 4-chlorobenzoic acid dehalogenase from *Pseudomonas* sp. CBS3 has been cloned on a 9.8 kbp fragment (Savard *et al.*, 1986). From deletion experiments it was suggested that this whole fragment was necessary for the expression of dehalogenating activity. Here we present evidence that only two closely linked much smaller DNA fragments, each expressing a single polypeptide chain, are necessary for this reaction.

Methods

Bacterial strains, plasmids and growth conditions. The bacteria and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C in L-broth (Maniatis *et al.*, 1982), containing 100 µg ampicillin ml⁻¹ when appropriate. *Pseudomonas* sp. CBS3 was grown

at 30 °C as described by Klages & Lingens (1979) on mineral salts medium containing 2 mM-4-chlorobenzoate.

Isolation of DNA and cloning procedures. Preparative amounts of plasmid DNA were prepared by the method of Birnboim & Doly (1979) with subsequent purification of the DNA by CsCl density-gradient centrifugation. For analytical purposes plasmids were isolated by the method of Holmes & Quigley (1981). Subcloning and restriction mapping were accomplished by established techniques (Maniatis *et al.*, 1982). DNA fragments were eluted from 0.7% agarose gels by the freeze-squeeze method of Tautz & Renz (1983). The ligated constructs were used to transform *E. coli* TG1 by the calcium chloride method (Mandel & Higa, 1970).

Preparation of crude extracts. *E. coli* cells were grown overnight in 1 litre of L-broth containing ampicillin. After centrifugation at 4 °C and 4000 g for 10 min, the cells were resuspended in 3 ml 50 mM-potassium phosphate buffer pH 7.0 and were passed twice through a French pressure cell at 35 MPa. Cell debris was removed by centrifugation at 4 °C and 40000 g for 30 min.

Enzyme assay. 4-Chlorobenzoate dehalogenase activity was assayed by quantitative thin-layer chromatography as described before (Thiele *et al.*, 1987). For activity tests with single components, the other components were added in excess.

Enrichment of the two cloned components of 4-chlorobenzoate dehalogenase from *E. coli* crude extracts. Crude extracts from *E. coli* clones, containing the cloned components of the 4-chlorobenzoate dehalogenase, were heated to 55 °C for 5 min. The resulting precipitates were removed by centrifugation at 4 °C and 40000 g for 30 min. The supernatants were then brought to 45% saturation with solid (NH₄)₂SO₄. After centrifugation the resulting pellets were dissolved in 3 ml 50 mM-potassium phosphate buffer pH 7.5 and applied to a Sephadex G150 column (2.5 × 100 cm). Proteins were eluted from the column with the same buffer. Fractions were collected and assayed for protein at 280 nm and tested for 4-chlorobenzoate dehalogenating activity.

Table 1. *Bacterial strains and plasmids used in this study*

Strain or plasmid	Phenotype	Reference
<i>Pseudomonas</i> sp. CBS3	4-chlorobenzoate dehalogenase	Klages & Lingens (1979)
<i>E. coli</i> TG1	LacZ ⁻	Gibson (1984)
<i>E. coli</i> DS910	Minicell strain; RecA ⁻ derivative of strain DS410	Dougan & Sherratt (1977)
pUC18	Ap ^r	Vieira & Messing (1977)
pPSA 843	Ap ^r , 4-chlorobenzoate dehalogenase	Savard <i>et al.</i> (1986)
pCBSII	Ap ^r	This study
pCBSIIIa	Ap ^r	This study
pCBSIIIb	Ap ^r	This study

Hybridization experiments. Chromosomal DNA from *Pseudomonas* sp. CBS3 and plasmid DNA of pCBSII and pCBSIII were digested with *Hind*III or *Sal*I and loaded on an agarose gel. DNA was blotted from the gel as described by Southern (1975) on a NY3N Nytran membrane (Schleicher & Schüll). The filter was hybridized with a nick-translated, biotinylated probe of pCBSII (Schneider & Müller, 1988). The nick-translation kit was purchased from Gibco Bethesda Research Laboratories; the biotin-16-dUTP was from Boehringer.

Identification of the gene products of the cloned fragments. For the identification of the protein products from the *E. coli* clones expressing component II and component III, we performed minicell experiments with strains harbouring pCBSII and pCBSIIIa according to Dougan & Sherratt (1977). [³⁵S]Methionine was purchased from Amersham.

Results

Separate cloning of the two components of 4-chlorobenzoate dehalogenase from Pseudomonas sp. CBS3

Savard *et al.* (1986), who cloned the 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3, suggested from their deletion experiments that all of the 9.8 kbp fragment was necessary for the dehalogenation reaction, and that the dehalogenase had to comprise several protein subunits. We therefore digested this 9.8 kbp insert with various restriction enzymes and ligated the fragments obtained into the plasmid pUC18 (Vieira & Messing, 1982). All of these subclones were grown in 1 litre cultures and crude extracts were prepared. As expected, none of these extracts showed dehalogenating activity. However, when the extracts of two clones, one containing a 3 kbp *Hind*III fragment (pCBSII) and the other containing a 2.6 kbp *Sal*I fragment (pCBSIIIa), were mixed, dehalogenating activity was observed. The 2.6 kbp *Sal*I fragment could be further shortened by digestion with *Pst*I to 1.3 kbp (pCBSIIIb). The subcloning procedure is outlined in Fig. 1.

Identification of the dehalogenase components encoded in the subclones

In previous biochemical studies, we found that 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3 comprised at least three different components (Elsner *et al.*, 1991). Components II and III were stable proteins, whereas component I was an unstable small molecule. When we mixed crude extract from the clone containing pCBSII with the enriched components of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3, only component III had to be added to restore dehalogenating activity. To crude extracts of the pCBSIII-containing clone only component II had to be added for activity. We therefore concluded that pCBSII encoded component II, whereas pCBSIIIa and pCBSIIIb coded for component III. The small component I seemed to be present in both extracts. However, when we used crude extracts from *E. coli* containing no plasmid at all, this extract also substituted for component I. Upon dialysis the extracts lost the ability to substitute for component I. We therefore concluded that component I was a small cofactor common in crude extracts of *E. coli*, which was not necessarily encoded in the two plasmids. In order to confirm the identities of the two cloned components, we partially purified the components from the crude extracts of the clones by heat treatment and ammonium sulphate precipitation. In these purification steps the component expressed from pCBSII behaved identically to component II and that from pCBSIII to component III of the 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3. In gel filtration experiments on Sephadex G150 the two components comigrated with the authentic components from *Pseudomonas* sp. CBS3 at molecular masses of 86 kDa and 92 kDa, respectively. The activity of component II in extracts of *E. coli* containing pCBSII was 2.9 mU (mg protein)⁻¹, whereas the activity of component

Fig. 1. Subcloning strategy of the 9.8 kbp insert encoding 4-chlorobenzoic acid dehalogenase from pPSA843. pCBSII encodes component II, pCBSIIIa and pCBSIIIb encode component III. H, *Hind*III; P, *Pst*I; S, *Sal*I.

III in the crude extracts of pCBSIIIa containing *E. coli* clones was 3.5 mU (mg protein)⁻¹. Both components were expressed constitutively in their clones.

Expression analysis of the cloned fragments by minicell experiments

In order to determine the size and number of polypeptide chains encoded in the cloned fragments, we performed minicell experiments according to Dougan & Sherratt (1977). The radioactively labelled gene products were detected by autoradiography after separation by SDS-polyacrylamide gel electrophoresis. In both clones only a single polypeptide was expressed from the DNA insert (Fig. 2). pCBSII coded for a 29 kDa polypeptide, whereas the polypeptide from pCBSIIIa was 31 kDa.

Restriction analysis and hybridization experiments

The restriction maps obtained from the two inserts (Fig. 1) suggested that the inserts from pCBSII and pCBSIIIa contain overlapping sequences. We therefore labelled the insert of pCBSII and performed Southern analysis on pCBSIIIa and *Pseudomonas* CBS3 chromosomal DNA. As shown in Fig. 3, the insert from pCBSIIIa hybridized with that from pCBSII. In the *Hind*III digests of chromosomal DNA from *Pseudomonas* CBS3 the two expected bands corresponding to the insert of pCBSII hybridized, whereas in the *Sal*I digests the band corresponding to the insert of pCBSIIIa hybridized, together with a second band at about 4.4 kb, containing the non-overlapping parts of the insert from pCBSII. From these results it is clear that the inserts from pCBSII and pCBSIIIa contained identical sequences.

Fig. 2. SDS-polyacrylamide gel electrophoresis of minicell extracts. Lane 1, pUC18; lane 2, pCBSII, which contains a 3.0 kbp insert coding for component II of 4-chlorobenzoate dehalogenase; lane 3, pCBSIIIa, which contains a 2.6 kbp insert coding for component III of 4-chlorobenzoate dehalogenase. The molecular masses of the standards used are indicated on the left.

Substitution of component I by ATP, coenzyme A and Mg²⁺

Recent results of F. Löffler (unpublished) suggested that in the reaction of 4-chlorobenzoate dehalogenase from *Pseudomonas* CBS3 component I could be replaced by a mixture of ATP, coenzyme A and Mg²⁺. When we used mixtures of dialysed inactive extracts from *E. coli* clones containing pCBSII and pCBSIIIb, the addition of 1 mM-ATP, 1 mM-coenzyme A and 1 mM-MgSO₄ restored the dehalogenating activity.

Discussion

The two protein components of 4-chlorobenzoate dehalogenase have been cloned and expressed separately in *E. coli*. Both components were expressed constitutively, which indicates that they are under negative control in the parent strain and that the regulatory sequences have not been cloned. In the original clone pPSA843 (Savard *et al.*, 1986) as well as in several clones we obtained from a genomic library we constructed from *Pseudomonas* sp.

Fig. 3. Southern blotting and hybridization of pCBSII, pCBSIIIa and chromosomal DNA from *Pseudomonas* sp. CBS3 with biotinylated pCBSII insert DNA. (a) Agarose gel used for Southern blotting; (b) Southern blot with biotinylated pCBSII as probe. Lanes: 1, plasmid DNA from pUC18 digested with *Hind*III; 2, plasmid DNA from pCBSIIIa digested with *Sal*I; 3, plasmid DNA from pCBSII digested with *Hind*III; 4, DNA from *Pseudomonas* sp. CBS3 digested with *Sal*I; 5, DNA from *Pseudomonas* sp. CBS3 digested with *Hind*III; 6, *Hind*III digested λ -DNA.

CBS3 (A. Elsner, unpublished results) both components were also expressed constitutively, which is in contrast to *Pseudomonas* sp. CBS3, where the expression of the enzyme is regulated by 4-chlorobenzoate. Since some of these clones contained inserts of more than 30 kbp the regulatory sequences must be located far from the structural genes. Another explanation might be that these regulatory sequences were not functional in *E. coli*.

The structural genes of the two components must be closely linked, as evidenced by the overlapping sequences in the clones expressing the two components. From these data it is clear that for the constitutive expression of 4-chlorobenzoate dehalogenase in *E. coli* only a much smaller DNA fragment was necessary than was originally suggested (Savard *et al.*, 1986).

None of the 4-chlorobenzoate dehalogenases described so far has been purified to homogeneity. Therefore, nothing is known about the subunit structure of the components involved in the dehalogenation reaction. From the gel filtration experiments and from the minicell experiments, we suggest that component II and component III are both comprised of three identical protein subunits.

The small unstable component I could be replaced by a mixture of coenzyme A, ATP and Mg^{2+} . The biosynthesis of these compounds is not located on the DNA fragments of the clones showing dehalogenating activity but the general availability of these compounds in crude extracts makes the dehalogenation reaction possible when the two protein components described above are expressed.

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