Genetic adaptation of bacteria to halogenated aliphatic compounds

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Chapter 6

The trans-3-chloroacrylic acid dehalogenase from *Pseudomonas pavonaceae* 170 shares structural and mechanistic similarities with 4-oxalocrotonate tautomerase

Gerrit J. Poelarends, Raymond Saunier, and Dick B. Janssen

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The genes (*caAD1* and *caAD2*) encoding the trans-3-chloroacrylic acid dehalogenase (CaaD) of the 1,3-dichloropropene-utilizing bacterium *Pseudomonas pavonaceae* 170 were cloned and heterologously expressed in *Escherichia coli* and *Pseudomonas* sp. strain GJ1. CaaD is a hexameric protein of 50 kDa that consists of three α-subunits of 75 amino acid residues and three β-subunits of 70 residues. It catalyzes the hydrolytic cleavage of the β-vinylic carbon-chlorine bond in trans-3-chloroacrylic acid with a turnover number of 6.4 s⁻¹. On the basis of sequence similarity, oligomeric structure, and subunit size, CaaD appears to be related to 4-oxalocrotonate tautomerase (4-OT). This tautomerase consists of six identical subunits of 62 amino acid residues and catalyzes the isomerization of 2-oxo-4-hexene-1,6-dioate, via hydroxymuconate, to yield 2-oxo-3-hexene-1,6-dioate. In view of the oligomeric architecture of 4-OT, a trimer of homodimers, CaaD is postulated to function as a trimer of αβ-dimers. The sequence conservation between CaaD and 4-OT and site-directed mutagenesis experiments suggested that Pro-1 of the β-subunit and Arg-11 of the α-subunit are active site residues in CaaD. Pro-1 could act as proton acceptor/donor and Arg-11 is probably involved in carboxylate binding. Based on these findings, a novel dehalogenation mechanism is proposed for the CaaD-catalyzed reaction which does not involve the formation of a covalent enzyme-substrate intermediate.
INTRODUCTION

Isomer-specific 3-chloroacrylic acid dehalogenases catalyze the hydrolytic cleavage of the β-vinyl carbon-chlorine bond in either cis- or trans-3-chloroacrylic acid to yield malonic acid semialdehyde and HCl. These enzymes are produced by both gram-positive and gram-negative bacteria, including Pseudomonas pavonaceae 170 (29), Pseudomonas cepacia CAA1 (12), and the coryneform bacterial strains FG41 (49) and CAA2 (12), enabling these organisms to use one or both isomers of the xenobiotic compound 3-chloroacrylic acid for growth. The dehalogenases from strain FG41 were purified to homogeneity and the trans-3-chloroacrylic acid dehalogenase was found to be a 50 kDa enzyme composed of different subunits of 8.7 and 7.4 kDa, whereas the cis-3-chloroacrylic acid dehalogenase was an enzyme composed of two or three identical 16 kDa subunits (49). Although large fragments of these dehalogenating enzymes were sequenced, no significant sequence similarities with other protein sequences were found when the different databases were searched in 1992 (49).

Whereas most hydrolytic dehalogenases that are active with halogenated aliphatic compounds (so-called halidohydrolases), such as haloalkane dehalogenases (28, 32, 52), haloacetate dehalogenases (16-18) and 2-haloacid dehalogenases (23, 27, 33), are only able to displace halogens bound to sp^3-hybridized carbon atoms, 3-chloroacrylic acid dehalogenases are unique in that they can cleave the much more stable vinylic carbon-halogen bond, in which the halogen is bound to an sp^2-hybridized carbon atom. Cleavage of the latter can also occur with 4-chlorobenzoyl-CoA dehalogenases, but in that case activation of the substrate (4-chlorobenzoate) to its coenzyme A derivative is needed (2, 6, 54). 3-Chloroacrylic acid dehalogenases are to our knowledge the only enzymes known to dehalogenate substrates with unactivated vinylic halogens.

Nothing is known about the catalytic mechanism of 3-chloroacrylic acid dehalogenases. To obtain insight in the structure, mechanism, and ancestry of these enzymes, we sequenced the genes encoding the trans-3-chloroacrylic acid dehalogenase of P. pavonaceae 170 and characterized the expressed protein. The results indicate that the dehalogenase shares both structural and mechanistic similarities with 4-oxalocrotonate tautomerase, an enzyme involved in the bacterial catabolism of catechol to metabolites in the Krebs cycle.

MATERIALS AND METHODS

Materials

All reagents, buffers, and solvents were obtained from Acros Organics, Merck, or
Sigma unless noted otherwise. Tryptone and yeast extract were obtained from Difco. Halogenated compounds were obtained from Acros Organics, Lancaster, Maybridge, or Aldrich, and were at least 97% pure according to the manufacturer. Restriction enzymes, Taq DNA polymerase, T4 DNA ligase, multipurpose agarose, the high pure plasmid isolation kit, and chemicals used for PCR amplification were purchased from Boehringer Mannheim. The QIAquick PCR purification kit and the QIAEX II gel extraction kit were obtained from Qiagen. DEAE-cellulose was obtained from Whatman Ltd. (Kent, England) and hydroxylapatite was obtained from Bio-Rad laboratories (Richmond, Calif.). A prepacked Superdex 75 gel filtration column and a low molecular weight gel filtration calibration kit were purchased from Pharmacia. Oligonucleotides were supplied by Eurosequence BV (Groningen, The Netherlands).

Bacterial strains, plasmids and growth conditions

The characteristics of P. pavonaceae 170, formerly known as Pseudomonas cichorii 170, are given elsewhere (29, 50). Escherichia coli JM101 (55) and plasmid pBluescript SK− (Stratagene) were used for subcloning experiments. E. coli HB101 (pRK600) (9) was the helper strain used for mobilizing pLAFR3-derived cosmids and pDSK519-derived plasmids in triparental matings with Pseudomonas sp. strain GJ1 (14). Cosmid pLAFR3 and plasmid pDSK519 are mobilizable broad-host-range vectors (19, 37). E. coli BL21(DE3) was used in combination with the T7 expression system (pET5a system; Promega) for overexpression of the dehalogenase and the mutant enzymes (42).

Cells for general cloning and expression were cultivated at 30°C in Luria-Bertani (LB) medium (35). When required, Difco agar (15 g/liter) was added to the medium. Antibiotics were added in the following amounts: ampicillin, 100 μg/ml; tetracycline, 12.5 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml.

General methods

Techniques for restriction enzyme digestion, ligation, transformation, and other standard molecular biology manipulations were based on methods described by Sambrook et al. (35). Triparental matings were carried out as described elsewhere (15). DNA sequencing was performed at the BioMedical Technology Centre (Groningen, The Netherlands) using a Pharmacia ALF-Express automatic sequencing machine according to the instructions provided with the Amersham Thermo Sequenase cycle-sequencing kit. The base sequence was determined by analyzing fluorescent dye-labeled nucleotide fragments. Nucleotide sequence data were analyzed by using the programs supplied in the DNASTAR software package (DNASTAR inc., Madison WI, USA). Searches for nucleotide and amino acid sequence similarities were done by using the BLAST program (1) and the DDBJ/EMBL/Genbank databases. Amino acid sequences were aligned by using CLUSTALW (48). Protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under
denaturing conditions on gels containing 15 - 20% polyacrylamide. The gels were stained with Coomassie brilliant blue. Protein concentrations were estimated with Coomassie brilliant blue by using bovine serum albumin as the standard. N-terminal amino acid sequencing was performed by Eurosequence BV using chemicals, reagents, and a sequenator (Model 477A) of Applied Biosystems (Warrington, UK). The native molecular mass of the purified dehalogenase was determined by Superdex 75 gel filtration using a FPLC system according to the instructions provided with the Superdex 75 column and the low molecular weight calibration kit of Pharmacia. Circular dichroism (CD) spectra were recorded on a AVIV CD spectrometer (62A DS).

**Enzyme activities**

Dehalogenase assays were performed by incubating an appropriate amount of enzyme or cell extract with 3 ml of 5 mM substrate in 50 mM Tris-sulfate buffer (pH 8.2) at 30°C. Halide liberation was monitored colorimetrically as described previously (20). All dehalogenase activities are expressed as units per milligram; 1 U was defined as the amount of enzyme that catalyzes the production of 1 µmol of halide per min. Enzyme assays were carried out twice, and the differences in specific activities were less than 10%.

Single colonies on agar plates were screened for dehalogenase activity by monitoring halide production upon incubation with *cis*- or *trans*-3-chloroacrylic acid as described previously (30).

**Polymerase chain reaction**

PCR reactions were carried out in a Progene DNA thermal cycler (New Brunswick Scientific Benelux B.V.). The amplification reaction mixtures (100 µl) contained standard *Taq* amplification buffer, 250 µM of each deoxyribonucleotide triphosphate, 100 ng of each primer, 100 ng template DNA, and 2 U of *Taq* DNA polymerase. The cycling parameters were 94°C for 5 min followed by 30 cycles of 94°C for 60 s, 58°C for 60 s, and 72°C for 90 s, with a final elongation step of 72°C for 10 min. The reaction mixtures were subjected to electrophoresis in 1% agarose gels, and PCR products were stained with ethidium bromide.

**Construction of expression vectors**

The dehalogenase expression vector pET44T2 was made using the overlap extension polymerase chain reaction as described by Ho *et al.* (13). The external PCR primers were oligonucleotides 5’-CACGGCATATGCGCATGATCTCTTGCAG-3’ (primer A) and 5’-TTGCCCAGCCAGAGCTAGCT-3’ (primer D). Primer A contains a *NdeI* restriction site (in bold) and anneals to the 5’ end of the *caad1* gene. Primer D contains a *BamHI* restriction site (in bold) and anneals to the complementary sequence directly downstream of the *caad2* gene. The internal PCR primers were oligonucleotides 5’-CATGTTATCTCTCCATTTGAGTT-3’ (primer B) and 5’- TCAAGTAATGAAGG-
MECHANISM OF *trans*-3-CHLOROACRYLIC ACID DEHALOGENASE

AGATAACATGCCCTTC-3’ (primer C). Primer C contains the desired mutations (underlined) that result in a new ribosome binding site (identical to the one provided by plasmid pET5a) in front of the *caaD2* gene. Primer B is the complementary primer. In two separate PCR’s, the AB and CD fragments were generated using cosmid pPS41, which harbors the dehalogenase genes, as the template with primers A and B in one reaction and primers C and D in a second reaction. The PCR reaction mixtures were subjected to electrophoresis in an 1% agarose gel, and the two PCR fragments were extracted separately using the QIAEX II gel extraction kit. Subsequently, a second PCR was carried out on a mixture of the AB and CD fragments using primers A and D. The mutated DNA fragment was isolated from an 1% agarose gel. The restriction sites *Nde*I and *Bam*HI were used to clone this DNA fragment into plasmid pET5a for overexpression of the dehalogenase under control of the T7 promoter. The newly constructed plasmid, pET44T2, was sequenced in order to verify the mutations in front of the *caaD2* gene.

A dehalogenase expression vector for *Pseudomonas* sp. strain GJ1 was constructed by cloning the *Sal*I fragment of cosmid pPS41 into the *Sal*I linearized broad-host-range vector pDSK519, resulting in pDSKcaaD. Upon introduction of this vector into *Pseudomonas* sp. strain GJ1, high level expression of the dehalogenase gene under control of its own promoter was obtained.

**Site-directed mutagenesis**

The *trans*-3-chloroacrylic acid dehalogenase mutants were constructed using the coding sequence for the dehalogenase in plasmid pET44T2 as the template. The αP1A, αR11A, and αR11K mutants were generated by PCR using the primers 5’-CACGGGATATGCGGCGATGATCTCTTGGCGAC-3’; 5’-CACGGGATATGCGGCGATGATCCTCTTGGCGACGTATGCCAGAACAAAAA-3’; and 5’-CACGGGATATGCGGCGATGATCTCTTGGCGACGTATGCCAGAACAAAAA-3’; respectively. These primers anneal to the 5’ end of the wild-type coding sequence and were used in combination with primer D. They all contain a *Nde*I restriction site (in bold) and the codon for the desired mutation (in italics). The αF39A, αF39Y, and βP1A mutants were generated by overlap extension PCR. Primers A and D were used as the external PCR primers. For the αF39A mutant, the internal PCR primers were oligonucleotides 5’-GAGCCCCGGAGAACATTGCCCGGTTTGGATT-3’ (mutated codon in italics) and 5’-AATGTCTTTCGCGGCGGCTC-3’ (primer E). For the αF39Y mutant, the internal PCR primers were oligonucleotide 5’-GAGCCCCGGAGAACATTGCCCGGTTTGGATT-3’ (mutated codon in italics) and primer E. For the βP1A mutant, the internal PCR primers were oligonucleotides 5’-TCAAGTAATGAAGGAGATAACATGGCCTCTTC-3’ (mutated codon in italics) and 5’-CATGTTATCTCCTTACTTACTTGGATT-3’.

PCR reactions were carried out as described above and PCR products were purified.
using the QIAquick PCR purification kit or the QIAEX II gel extraction kit. The restriction sites \( NdeI \) and \( BamHI \) that were introduced during the amplification reactions were used to clone the PCR products into plasmid pET5a for overexpression of the dehalogenase mutants. The cloned dehalogenase genes were sequenced in order to verify the mutations.

**Preparation of crude extracts**

The \( \text{trans-3-chloroacrylic acid dehalogenase} \) and the mutant enzymes were expressed in \( E. coli \) BL21(DE3) using the pET system. Fresh BL21(DE3) transformants containing the desired plasmid were collected from a plate by resuspending them in 1 ml of LB medium and were used to inoculate 100 ml of LB/ampicillin medium to a starting \( OD_{600} \) of 0.1. After overnight growth at 30\( ^\circ \)C, cells were harvested by centrifugation (10 min at 10,000 \( \times g \)), washed with 1 volume of 50 mM Tris-sulfate buffer (pH 8.2), and disrupted at 4\( ^\circ \)C in an appropriate amount of this buffer by sonication (10 s per ml of suspension at a 70 W output in a Vibra cell sonicator). A crude extract was obtained by centrifugation (45 min at 16,000 \( \times g \)).

**Purification of the dehalogenase**

For isolation of the \( \text{trans-3-chloroacrylic acid dehalogenase} \) of \( P. \) pavonaceae 170, a single colony of strain 170 was used to inoculate 100 ml of LB medium. After overnight growth at 30\( ^\circ \)C, the culture was used to inoculate 1 liter of LB medium. This culture was grown at 30\( ^\circ \)C until the early stationary growth phase. Cells were harvested by centrifugation (10 min at 10,000 \( \times g \)), washed with 1 volume of TEMAG buffer (10 mM Tris-SO\(_4\), 1 mM EDTA, 1 mM \( \beta \)-mercaptoethanol, 0.02% sodium azide, 10% glycerol, pH 8.0), and stored at -20\( ^\circ \)C until further use. Preparation of a crude extract and purification of the dehalogenase were done as described below for the recombinant enzyme.

High level \( \text{trans-3-chloroacrylic acid dehalogenase} \) expression was obtained in \( Pseudomonas \) sp. strain GJ1. A single colony of strain GJ1 containing the expression vector pDSKcaaD was used to inoculate 10 ml of LB/kanamycin medium. After overnight growth at 30\( ^\circ \)C, the culture was used to inoculate 1 liter of LB/kanamycin medium. This culture was also grown overnight at 30\( ^\circ \)C. Cells were harvested by centrifugation (10 min at 10,000 \( \times g \)), washed with 1 volume of TEMAG buffer, and stored at -20\( ^\circ \)C.

The \( \text{trans-3-chloroacrylic acid dehalogenase} \) was purified to homogeneity by using a modification of a published procedure (49). In a typical experiment, cells of a 1 liter-culture were thawed and suspended in 20 ml of TEMAG buffer. The cells were disrupted at 4 to 10\( ^\circ \)C by continuous sonication, after which unbroken cells and debris were removed by centrifugation for 1 h at 50,000 rpm in a type 70 Ti rotor (Beckman). The supernatant was applied to a DEAE-cellulose column which had previously been equilibrated with TEMAG buffer. The column was washed with 1 column volume of TEMAG buffer, and the proteins were eluted with a linear gradient of 0 to 0.5 M ammonium sulfate in TEMAG. Fractions that
showed the highest dehalogenase activity with \textit{trans}-3-chloroacrylic acid were pooled and dialyzed overnight against PEMAG buffer (5 mM potassium phosphate, 1 mM EDTA, 1 mM \(\beta\)-mercaptoethanol, 0.02% sodium azide, 10% glycerol, pH 6.5). The dialysate was loaded onto a hydroxylapatite column which had previously been equilibrated with PEMAG buffer. The column was washed with 1 column volume of PEMAG buffer, and the proteins were eluted with a linear gradient of 5 to 100 mM potassium phosphate in PEMAG. Fractions with the highest \textit{trans}-3-chloroacrylic acid dehalogenase activity were analyzed by SDS-PAGE, and those that contained purified enzyme were pooled and dialyzed against TEMAG buffer. The enzyme was stored at 4°C or -20°C.

**Nucleotide sequence accession number**

The nucleotide sequence of the dehalogenase gene region has been deposited in the Genbank database under accession number AJ290446.

**RESULTS**

**Cloning and characterization of the genes encoding the \textit{trans}-3-chloroacrylic acid dehalogenase**

The \textit{trans}-specific 3-chloroacrylic acid dehalogenase of \textit{P. pavonaceae} 170 was partially (~75%) purified. After SDS-PAGE (data not shown), two dominant protein bands at about 7.5 and 8.5 kDa were observed, indicating that the dehalogenase consisted of two different subunits. Both subunits were subjected to N-terminal sequence analysis. The sequence of the 8.5 kDa subunit (designated \(\alpha\)-subunit) was established as P-M-I-S-C-D-M-R-Y-G-R-T-D-E-Q-K and that of the 7.5 kDa subunit (designated \(\beta\)-subunit) as P-F-I-E-C-H-I-A-T-G-L-S-V-A-R-K-Q-Q-L-I-R-D.

To isolate the genes encoding the \textit{trans}-3-chloroacrylic acid dehalogenase, individual clones of a previously constructed cosmid library of \textit{P. pavonaceae} 170 (31) were screened for dehalogenase activity by monitoring halide production upon incubation with \textit{trans}-3-chloroacrylic acid. Out of 2,500 \textit{E. coli} HB101 clones tested, 1 clone expressing the \textit{trans}-3-chloroacrylic acid dehalogenase was found. The recombinant cosmid (pPS41) encoding the dehalogenase was isolated and the localization of the dehalogenase genes was determined by screening subclones for dehalogenase activity.

Nucleotide sequencing of a 2.6 kb \textit{SalI} subclone of pPS41 revealed several open reading frames (Fig. 1). The two open reading frames encoding the \textit{trans}-3-chloroacrylic acid dehalogenase (designated \textit{caaD1} and \textit{caaD2}) were identified by using the N-terminal amino acid sequences determined for both subunits of the enzyme isolated from \textit{P. pavonaceae} 170. These N-terminal amino acid sequences are identical to those predicted from the DNA
sequence if the initiating N-formyl-methionines are removed during posttranslational processing. The \textit{caad1} gene encodes a protein (the $\alpha$ subunit) of 76 amino acids with a calculated molecular weight (M,\textsubscript{r}) of 8.47 kDa. The \textit{caad2} gene was found downstream of \textit{caad1} and encodes a protein (the $\beta$ subunit) of 71 amino acids (M,\textsubscript{r}, 7.64 kDa). The M,\textsubscript{r}’s of both subunits are in agreement with their masses estimated from SDS-PAGE.

Screening of the cosmid library of \textit{P. pavonaceae} 170 did not reveal clones that expressed the \textit{cis}-3-chloroacrylic acid dehalogenase. To check for the occurrence of a \textit{cis}-3-chloroacrylic acid dehalogenase gene or other genes related to 3-chloroacrylic acid utilization in the vicinity of the \textit{caad1} and \textit{caad2} genes, the DNA regions flanking these genes were analyzed. An open reading frame, designated \textit{orf130}, encoding a putative protein of 130 amino acids was found downstream of \textit{caad2} (Fig. 1). Orf130 shares considerable similarity with the hypothetical proteins Orf129 (53\% identity) and YodA (51\% identity) of \textit{Bacillus subtilis} (22). However, the possible functions of these hypothetical proteins have not been reported. Orf130 also shares notable similarities with the \textit{caad1} gene product including an N-terminal proline and a short stretch of sequence, YGRTD(A,E)Q, which they have in common. This notion prompted us to test if the \textit{orf130} gene codes for the \textit{cis}-3-chloroacrylic acid dehalogenase. Crude extract of \textit{E. coli} cells overexpressing Orf130 did not show dehalogenase activity toward \textit{cis}-3-chloroacrylic acid. Orf130 was also not capable of dehalogenating \textit{trans}-3-chloroacrylic acid nor of converting \textit{cis}-3-chloroacrylic acid into \textit{trans}-3-chloroacrylic acid. These results indicate that the \textit{orf130} gene product is not involved in 3-chloroacrylic acid utilization. Downstream of \textit{orf130} the beginning of an open reading frame (\textit{orf4}) was found that codes for a peptide of 178 amino acids that shares extensive similarity with the N-termini of the putative alcohol dehydrogenases YhdH (56\% identity) of \textit{E. coli} (3) and YhfP (47\% identity) of \textit{B. subtilis} (22). In the 1 kb region upstream of the \textit{caad1} gene, no open reading frames encoding putative proteins with significant similarity to protein sequences present in the current databases were found.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Organization of the \textit{trans}-3-chloroacrylic acid dehalogenase gene region of \textit{P. pavonaceae} 170. Genes are shown as open boxes and arrows indicate the direction of transcription. \textit{SalI} restriction sites (S) are shown.}
\end{figure}
Expression and characterization of the dehalogenase

E. coli HB101 harboring recombinant cosmid pPS41 displayed poor expression of the trans-3-chloroacrylic acid dehalogenase (Table 1). Introduction of pPS41 into Pseudomonas sp. strain GJ1 resulted in significantly better expression (Table 1). To obtain overexpression of the dehalogenase in strain GJ1, we cloned the SalI fragment that harbors the dehalogenase genes (Fig. 1) into the Pseudomonas high-copy number vector pDSK519, resulting in pDSKcaaD. The coding sequence for the dehalogenase in pDSKcaaD is under control of its own promoter and the dehalogenase was expressed constitutively in strain GJ1 in a soluble and active form up to a concentration equivalent to 13 % of the total soluble cellular protein (Table 1). The dehalogenase was isolated from strain GJ1(pDSKcaaD) with a yield of 20 mg of pure protein, as judged by SDS-PAGE, from a 1-liter culture. The purified enzyme could be stored for several months in TEMAG buffer at 4°C or -20°C without significant loss of activity.

Table 1. Expression of the trans-3-chloroacrylic acid dehalogenase in E. coli and Pseudomonas

<table>
<thead>
<tr>
<th>Organism</th>
<th>Dehalogenase sp acta (mU/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
</tr>
<tr>
<td>HB101 (pPS41)</td>
<td>30</td>
</tr>
<tr>
<td>BL21(DE3) (pET44T2)</td>
<td>1500</td>
</tr>
<tr>
<td><strong>Pseudomonas strains</strong></td>
<td></td>
</tr>
<tr>
<td><em>P. pavonaceae</em> 170</td>
<td>310</td>
</tr>
<tr>
<td>GJ1 (pPS41)</td>
<td>400</td>
</tr>
<tr>
<td>GJ1 (pDSKcaaD)</td>
<td>2380b</td>
</tr>
</tbody>
</table>

aSpecific activities for trans-3-chloroacrylic acid (5 mM) were determined with crude extracts prepared from cells grown in Luria Broth.
bThe specific activity measured in crude extract from strain GJ1(pDSKcaaD) corresponds to a dehalogenase concentration equivalent to 13% of the total soluble cellular protein, based on a specific activity of 18,000 mU/mg for the purified enzyme.

The native molecular mass of the dehalogenase was estimated by gel filtration chromatography to be 50 kDa. A comparison of this value to the subunit molecular masses (8.47 kDa and 7.64 kDa) determined from the primary amino acid sequences suggests that the native dehalogenase is a hexameric protein, probably consisting of three α- and three β-
subunits ($\alpha_3\beta_3$).

The dehalogenase catalyzed the liberation of halide from trans-3-chloroacrylic acid (specific activity of 18 U/mg of protein) and trans-3-bromoacrylic acid (48 U/mg), but showed no detectable dehalogenase activity (<0.005 U/mg) toward cis-3-chloroacrylic acid, indicating that the enzyme is completely isomer-selective. The saturated analog 3-chloropropionic acid was also not dehalogenated, indicating that the halogen atom is only removed when attached to an unsaturated carbon atom. Since no activity was found for 2-bromoacrylic acid and 2-chloroacrylic acid, it is essential that the halogen substituent is located at the $\beta$-position. The enzyme was also not active with trans-3-chloroallyl alcohol and trans-1,3-dichloropropene, indicating the importance of the presence of a carboxyl group in the substrate.

The dehalogenase showed a broad pH optimum around 8.5 and the temperature optimum was 40°C. By measuring the initial velocities of product formation at different trans-3-chloroacrylic acid concentrations, a $K_m$ of 0.19 mM and $k_{cat}$ of 6.4 s$^{-1}$ were found.

Sequence similarity with 4-oxalocrotonate tautomeres/isomerases

Database searches identified seven related proteins as sharing significant sequence similarity with the trans-3-chloroacrylic acid dehalogenase (CaaD) (Table 2). Two are well-studied enzymes involved in the bacterial catabolism of catechol to metabolites in the Krebs cycle, the 4-oxalocrotonate tautomerase (4-OT) from Pseudomonas putida mt-2 (41) and the 73% identical isozyme from Pseudomonas sp. CF600 (43). Both are hexameric proteins that consist of identical subunits of 62 amino acid residues (5) and catalyze the isomerization of 2-oxo-4-hexene-1,6-dioate, via hydroxymuconate, to yield 2-oxo-3-hexene-1,6-dioate (53). The other five proteins that were retrieved from the similarity search have not been studied, but based on sequence similarity to 4-OT they might be classified as putative 4-oxalocrotonate tautomeres/isomerases. Pairwise identities among the seven identified 4-OT homologues range from 35 to 92%.

Alignments optimizing identity between the amino acid sequences of the two dehalogenase subunits and the seven 4-OT homologues are shown in Fig. 2. Pairwise identities between the $\alpha$-subunit (CaaD1) of the dehalogenase and the 4-OT sequences fall between 23 and 35% (Table 2), with the highest sequence similarity in the N-terminal region, particularly in the region boxed in Fig. 2A. Pairwise identities between the $\beta$-subunit (CaaD2) and the 4-OT sequences are lower, and range from 16 to 25% (Table 2).

The sequence alignment of Fig. 2 suggests catalytic residues for CaaD that are in the same position in the alignment as the identified catalytic residues of 4-OT. Affinity labeling (38), kinetic analysis (39), chemical synthesis (10), NMR (40, 41), site-directed mutagenesis (7), and crystallographic studies (43) identified the amino-terminal proline as the catalytic base in the 4-OT-catalyzed tautomerization reaction. This N-terminal proline is invariant
among all identified 4-OT homologues (Fig. 2). Both dehalogenase subunits also possess an N-terminal proline, indicating that one of these prolines may serve as the catalytic base in the CaaD-catalyzed dehalogenation reaction. The second catalytic residue of 4-OT, Arg-11, which is absolutely conserved among the 4-OT homologues (Fig. 2), was proposed to interact with the 6-carboxylate of the substrate (2-oxo-4-hexene-1,6-dioate) to facilitate both substrate binding and catalysis (11, 43). The sequence similarity indicates that Arg-11 in the α-subunit of the dehalogenase may perform an analogous role by interacting with the carboxylate group of trans-3-chloroacrylic acid. The third catalytic residue of 4-OT, Arg-39, which was proposed to interact with the 1-carboxylate and the 2-keto group of the substrate to promote carbonyl polarization and catalysis (11, 43), is not conserved in the dehalogenase sequence. This seems plausible since the dehalogenase substrate contains only one carboxylate group.

### Table 2. Protein sequences that are similar to trans-3-chloroacrylic acid dehalogenase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
<th>Length (aa)</th>
<th>Organism</th>
<th>Identity to α-subunit</th>
<th>Identity to β-subunit</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-OT</td>
<td>xylH</td>
<td>62</td>
<td>P. putida mt-2</td>
<td>26%</td>
<td>25%</td>
<td>Q01468</td>
</tr>
<tr>
<td>4-OT</td>
<td>dmpI</td>
<td>62</td>
<td>Pseudomonas sp. CF600</td>
<td>26%</td>
<td>22%</td>
<td>P49172</td>
</tr>
<tr>
<td>4-OT</td>
<td>ywhB</td>
<td>61</td>
<td>B. subtilis 168</td>
<td>35%</td>
<td>16%</td>
<td>CAB02512</td>
</tr>
<tr>
<td>4-OT</td>
<td>nahJ</td>
<td>62</td>
<td>P. stutzeri AN10</td>
<td>26%</td>
<td>24%</td>
<td>AAD02155</td>
</tr>
<tr>
<td>4-OT</td>
<td>xylH</td>
<td>79</td>
<td>S. aromaticivorans F199</td>
<td>25%</td>
<td>22%</td>
<td>AAD03991</td>
</tr>
<tr>
<td>4-OT</td>
<td>phnL</td>
<td>76</td>
<td>Pseudomonas sp. DJ77</td>
<td>23%</td>
<td>24%</td>
<td>AAD03836</td>
</tr>
<tr>
<td>4-OT</td>
<td>nahJ</td>
<td>62</td>
<td>P. putida G7</td>
<td>26%</td>
<td>22%</td>
<td>AAD13221</td>
</tr>
</tbody>
</table>

*Pairwise identities between the α- or β-subunit of the dehalogenase and the 4-OT sequences were calculated by using CLUSTALW.

### Characterization of dehalogenase mutants

In the homohexameric (α6) 4-OT molecule, Pro-1 is important for tautomerase activity since it serves as the catalytic base. To test if both Pro-1 in the α-subunit and Pro-1 in the β-subunit of the heterohexameric (α3β3) CaaD molecule are important for dehalogenase activity, each proline was replaced by an alanine. The wild type and mutant enzymes were expressed in E. coli BL21(DE3) and their specific activities with trans-3-chloroacrylic acid and trans-3-bromoacrylic acid were measured in crude extracts. Mutation of Pro-1 to alanine in the β-subunit essentially abolished catalytic activity of the dehalogenase, whereas mutation of Pro-1 to alanine in the α-subunit had no significant influence on activity (Table 3). To
determine whether the loss of catalytic activity in the βP1A mutant is due to the specific alteration of the catalytic residue or to the loss of native-like protein structure, this mutant was purified to homogeneity and analyzed by gel filtration chromatography and circular dichroism (CD). The purified βP1A mutant showed an activity of 10 mU/mg of protein with trans-3-chloroacrylic acid, which is 1800-fold lower than the activity of wild-type enzyme. The CD spectrum of this mutant was nearly identical to that recorded for wild type, indicating that the mutation did not result in any gross conformational change (data not shown). It was further shown by gel filtration chromatography that the native molecular mass for mutant βP1A is comparable to that of wild type, indicating that the hexameric association was still intact. Hence, the 1800-fold decrease in activity found with the βP1A mutant is probably due to a direct effect of the substitution of the catalytically important proline for alanine. Taken together, the results indicate that of the two N-terminal prolines in the dehalogenase, only the N-terminal proline in the β-subunit is important for the dehalogenase-catalyzed reaction.

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**Figure 2.** Alignments of the amino acid sequences of the α-subunit (CaaD1) or β-subunit (CaaD2) of the trans-3-chloroacrylic acid dehalogenase (CaaD) with the seven 4-OT sequences (A, B), or with the amino-terminal sequences of the subunits of the trans-3-chloroacrylic acid dehalogenase isolated from strain FG41 (C). Residues conserved throughout all sequences are indicated by an asterisk. Dashes represent residues absent in other sequences. The catalytically important residues in 4-OT are marked with + and are shown in boldface. The region of highest sequence identity among CaaD1 and the 4-OT sequences is boxed.
MECHANISM OF trans-3-CHLOROACRYLIC ACID DEHALOGENASE

Table 3. Activities of wild type and mutant 3-chloroacrylic acid dehalogenase for trans-3-chloroacrylic acid (CAA) and trans-3-bromoacrylic acid (BAA)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CAA (mU/mg of protein)</th>
<th>BAA (mU/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1500</td>
<td>3250</td>
</tr>
<tr>
<td>αP1A</td>
<td>1260</td>
<td>3050</td>
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<td>βP1A</td>
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<tr>
<td>αR11A</td>
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<td>240</td>
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<tr>
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<td>350</td>
<td>480</td>
</tr>
<tr>
<td>αF39Y</td>
<td>240</td>
<td>300</td>
</tr>
</tbody>
</table>

*Activities were measured in cell free extracts containing similar amounts of dehalogenase. Halide production with 5 mM substrate was determined at 30°C and pH 8.2.

Although CaaD has a low overall identity to the know 4-OTs, the region around Arg-11 is highly conserved (Fig. 2A). In 4-OT, this region is related to binding of the carboxylate group of the substrate (see above). The dehalogenase substrate also contains a carboxylate group, probably providing the evolutionary pressure to preserve this site in CaaD. To probe the role of Arg-11 in the catalytic activity of CaaD, this residue was mutated to alanine or lysine. Mutation of Arg-11 to alanine resulted in an inactive enzyme, indicating that Arg-11 is essential for dehalogenase activity (Table 3). The mutant αR11K had partially restored catalytic activity compared to the catalytically inactive αR11A mutant, suggesting that a positive charge at this position is important for carboxylate binding.

In many reactions involving carbon-halogen bond cleavage, the carbon-halogen bond is weakened by functional groups that interact with the halogen substituent (28, 34, 51, 52). In the α-subunit of the trans-3-chloroacrylic acid dehalogenase, Phe-39 is in the same position in the alignment as the catalytically important Arg-39 of 4-OT, suggesting that Phe-39 may be one of the residues that promotes carbon-halogen bond cleavage by interacting with the chlorine atom of the dehalogenase substrate. To test if Phe-39 is catalytically important, this residue was mutated to alanine or tyrosine. The αF39A and αF39Y mutants were still able to catalyze halide release from both dehalogenase substrates, although 5- to 10-fold slower compared to the wild type enzyme (Table 3), indicating that Phe-39 is not essential for dehalogenase activity.
DISCUSSION

The enzyme \textit{trans}-3-chloroacrylic acid dehalogenase (CaaD) is elaborated by the soil bacterium \textit{P. pavonaceae} 170 as part of a degradative pathway for the xenobiotic nematocide \textit{trans}-1,3-dichloropropene (29). This hydrolytic dehalogenase, of which the properties are reported in this work, shares no sequence similarity with other halidohydrolases but appears to be related to the family of 4-oxalocrotonate tautomeras. No other bacterial 3-chloroacrylic acid dehalogenase genes have been cloned, but the N-terminal sequences of the subunits of the \textit{trans}-3-chloroacrylic acid dehalogenase isolated from the gram-positive coryneform bacterial strain FG41 (49) share extensive similarity with the N-terminal parts of the CaaD subunits (Fig. 2C), suggesting that these two proteins have a common evolutionary origin. As might be expected, the two \textit{trans}-3-chloroacrylic acid dehalogenase sequences are more related to each other than to the 4-oxalocrotonate tautomeras.

The primary amino acid sequence of 4-oxalocrotonate tautomerase (4-OT) shows no apparent similarity with those of the mammalian enzymes D-dopachrome tautomerase (DDT) (44) and macrophage migration inhibitory factor (MIF) (45), nor with that of the bacterial enzyme 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) (43), but, remarkably, these four proteins share a common structural architecture (26, 44). DDT, MIF and CHMI have an almost identical subunit topology with two $\beta\alpha\beta$ motifs related by pseudo-2-fold symmetry and trimeric $\beta$-sheet packing (26, 43, 44). While CHMI, MIF, and DDT are functional as homotrimers, 4-OT is a hexamer of identical monomers. The 4-OT subunit is composed of only 62 residues and is dimerized by 2-fold symmetry to form a structure similar to that of the CHMI, MIF and DDT monomer. Therefore, 4-OT is a trimer of homodimers that shows 32 symmetry; its overall hexameric structure is very similar to the trimeric structure of CHMI, MIF, and DDT (43-45). An interesting difference between the four structures is that because of the higher symmetry of the 4-OT hexamer there are potentially six active sites in 4-OT, yet only three are conserved in CHMI, MIF, and DDT (43, 44).

One of the characteristics of this superfamily of 4-OT related proteins is that its members possess an amino-terminal proline that is located at the bottom of a hydrophobic pocket. CHMI and 4-OT utilize this proline as a catalytic base in their isomerization reactions (7, 10, 38-41, 43). Pro-1 of MIF is required for its D-dopachrome tautomerase and phenylpyruvate tautomerase activities (24, 36). The N-terminal proline of DDT is proposed to serve as the catalytic base in the DDT-catalyzed tautomerization reaction (44). The Pro-1 residue is conserved among all known homologues of 4-OT (Fig. 2), MIF (24, 46), and DDT (44), and is also conserved in both subunits of CaaD (Fig. 2). In the \textit{trans}-3-chloroacrylic acid dehalogenase isolated from strain FG41, however, the amino-terminal proline is present only in the $\beta$-subunit (Fig. 2C). This suggests that Pro-1 of the $\beta$-subunit may serve as a catalytic base in both of the \textit{trans}-3-chloroacrylic acid dehalogenating enzymes. Site-directed
mutagenesis experiments in which the amino-terminal prolines in CaaD were replaced by alanines indeed demonstrated that Pro-1 of the β-subunit is catalytically important, whereas Pro-1 of the α-subunit does not seem to play a role in catalysis.

On the basis of its sequence similarity to 4-OT, we conclude that CaaD also belongs to the superfamily of 4-OT related proteins (26). The relatedness between CaaD and 4-OT is most apparent from the presence of a short stretch of sequence, GR(T,S)DEQK, that they have in common (Fig. 2A). This sequence motif, which includes the catalytically important Arg-11 in both 4-OT and CaaD, is not conserved in MIF and DDT, but is present as GRSIEsrr around the equivalent, catalytically important, Arg-71 residue in CHMI. This functional motif is related to binding of the carboxylate group of the 4-OT, CHMI, and CaaD substrates.

In view of the oligomeric architecture of 4-OT, a trimer of homodimers (43), CaaD is postulated to function as a trimer of αβ-dimers. In contrast to the presence of potentially six active sites in the highly symmetrical 4-OT molecule (47), there are three potential active sites in CaaD. From the crystal structure of 4-OT inactivated by 2-oxo-3-pentynoate (47), the substrate should interact with Pro-1 of one subunit and Arg-11 from an adjacent subunit within the same homodimer. Consistent with this, the catalytically important Pro-1 of CaaD is located on the β-subunit, whereas Arg-11 is located on the α-subunit, showing that residues from both subunits contribute to the dehalogenase active site.

In 4-OT, Arg-11 interacts with the 6-carboxylate of the substrate (2-oxo-4-hexene-1,6-dioate) to facilitate substrate binding and catalysis, and Pro-1 transfers protons from C-3 to C-5 (Fig. 3A). We propose similar roles for Pro-1 and Arg-11 in CaaD, which leads to a minimal catalytic mechanism for the CaaD-catalyzed hydrolytic dehalogenation of trans-3-chloroacrylic acid (Fig. 3B). In principle, one can write several possible chemical pathways for the CaaD-catalyzed reaction, including a nucleophilic substitution and an addition-elimination mechanism. Although a one-step displacement of the halide by a hydroxyl ion has been suggested for the hydrolytic dehalogenation of D- and L-2-haloalkanoic acids by haloacid dehalogenase (DL-DEX 113) from Pseudomonas sp. 113 (27), it seems unlikely with the halide bound to an sp²-hybridized carbon atom as in trans-3-chloroacrylic acid. Therefore, we propose that, in parallel to the hydration of monofluorofumarate by fumarase (25), CaaD catalyzes nucleophilic addition of a hydroxyl group on an sp²-hybridized carbon atom. This nucleophilic addition is favoured over an electrophilic reaction because of the presence of the electron-withdrawing halogen and the carboxyl group. In this scenario, Pro-1 serves as the catalytic base that activates a water molecule to attack the β-carbon atom of the substrate. This leads to two hypothetical pathways, one involving the formation of the intermediate 3-chloro-3-hydroxypropanoic acid (route 1 in Fig. 3B) and the other involving the formation of a carbanion intermediate (route 2 in Fig. 3B). Product formation from either intermediate involves redirection of electrons with departure of Cl⁻ and protonation at C-2. The latter suggests the presence of an acidic residue, which may be the protonated proline or another
amino acid. Loss of a proton from the hydroxyl at C-3 would produce the malonic acid semialdehyde, which may be facilitated by water or another proton acceptor. Hydration of monofluorofumarate by fumarase also yielded an unstable intermediate, α-fluorohydrin (α-fluoromalate), which subsequently decomposes to oxaloacetate and HF (25).

Because CaaD catalyzes a dehalogenation reaction, it is anticipated that functional groups involved in halogen/halide binding are required in addition to Pro-1 and Arg-11. We speculate that Phe-39 in CaaD, which is in the same position in the alignment as Arg-39 in 4-OT (Fig. 2), may interact with the chlorine atom of the substrate to promote carbon-halogen bond cleavage. Indeed, aromatic ring systems are known to be partially positively charged in the plane of the ring (4). Phenylalanine residues were also proposed to contribute to halogen/halide binding in haloalkane dehalogenase (DhlA) and L-2-haloacid dehalogenase (DhlB) from *X. autotrophicus* GJ10 (8, 34). However, the F39A and F39Y mutants of CaaD still had some residual activity, indicating that this residue is not essential. The presence of other functional groups interacting with the halogen atom of the substrate could explain why these mutants retained some activity. Indeed, in DhlA and DhlB the halogen/halide binding site is formed by more than one residue (21, 34, 51).

**Figure 3.** A comparative reaction scheme for 4-oxalocrotonate tautomerase (4-OT) and *trans*-3-chloroacrylic acid dehalogenase (CaaD). The primed residues come from other subunits. A, the reaction catalyzed by 4-OT (adopted from references 11 and 43). B, the two proposed reaction schemes for CaaD, one involving the formation of 3-chloro-3-hydroxypropanoic acid (route 1) and the other involving the formation of a carbanion intermediate (route 2). Pro-1 is shown as the catalytic base to activate a water molecule that attacks the substrate. Arg-11 is implicated in substrate binding.
Screening of the cosmid library of *P. pavonaceae* 170 did not reveal clones that expressed the *cis*-3-chloroacrylic acid dehalogenase. Thusfar, the only sequence information available for *cis*-specific 3-chloroacrylic acid dehalogenases is the N-terminal sequence of the enzyme isolated from the coryneform bacterial strain FG41 (49). This enzyme is probably a trimeric protein of 16.2 kDa subunits, and a comparison of its amino-terminal sequence with those of the *trans*-3-chloroacrylic acid dehalogenases from strains FG41 and 170 revealed no overall similarity, but showed that Pro-1 and Arg-11 are conserved (data not shown). Therefore, *cis*- and *trans*-specific 3-chloroacrylic acid dehalogenases may catalyze the dehalogenation of their respective 3-chloroacrylic acid isomers through the same mechanism as shown in Fig. 3. This mechanism is different from that of most other hydrolytic dehalogenases in that it does not involve the formation a covalent enzyme-substrate intermediate.

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