**trans-3-Chloroacrylic Acid Dehalogenase from Pseudomonas pavonaceae 170 Shares Structural and Mechanistic Similarities with 4-Oxalocrotonate Tautomerase**

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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 protein of 50 kDa that is composed of α-subunits of 75 amino acid residues and 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 be a hexameric protein that functions 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 the 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.

Isomer-specific 3-chloroacrylic acid dehalogenases catalyze the hydrolytic cleavage of the β-vinylic 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 (27), *Pseudomonas cepacia* CAA1 (11), and the coryneform bacterial strains FG41 (47) and CAA2 (11), 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 *trans*-3-chloroacrylic acid dehalogenase (CaaD) 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 (47). 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 (47).

Whereas most hydrolytic dehalogenase that are active with halogenated aliphatic compounds (so-called halohydrolases), such as haloalkane dehalogenases (26, 30, 50), haloacetate dehalogenases (15–17), and 2-haloacid dehalogenases (21, 25, 31), are only able to displace halogens bound to sp²-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 sp²-hybridized carbon atom. Cleavage of the latter can also occur with 4-chlorobenzoyl-coenzyme A dehalogenases, but in that case activation of the substrate (4-chlorobenzoylate) to its coenzyme A derivative is needed (2, 5, 52). 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 on the structure, mechanism, and ancestry of these enzymes, we sequenced the genes encoding CaaD of *P. pavonaceae* 170 and characterized the expressed protein. The results indicate that the dehalogenase has both structural and mechanistic similarities to 4-oxalocrotonate tautomerase (4-OT), an enzyme involved in the bacterial catabolism of catechol to metabolites in the Krebs cycle.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The characteristics of *P. pavonaceae* 170, formerly known as *Pseudomonas cichorii* 170, have been given elsewhere (27, 48). *Escherichia coli* JM101 (53) and plasmid pBluescript SK− (Stratagene) were used for subcloning experiments. *E. coli* HB101(pRK600) (8) was the helper strain used for mobilizing pLAFR3-derived cosmids and pDSK519-derived plasmids in triparental matings with *Pseudomonas* sp. strain GJ1 (13). Cosmid pLAFR3 and plasmid pDSK519 are mobilizable broad-host-range vectors (18, 35).

**General methods.** Techniques for restriction enzyme digestion, ligation, transformation, and other standard molecular biology manipulations were based on methods described by Sambrook et al. (33). Triparental matings were carried out at 30°C in Luria-Bertani (LB) medium (33). 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; and kanamycin, 50 μg/ml.

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as described elsewhere (14). 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 analyzed using an Nd6 restriction site (in bold) and the codon for the desired mutation (in italics). The f39A, f39Y, and P1A mutants were generated by overlap extension PCR. Primers A and D were used as the external PCR primers. For the f39A mutant, the internal PCR primers were oligonucleotides 5′-GA GCCGGCGGAGAACATTGCCTGATT-3′ (mutated codon in italics) and 5′-AATTGTCTCCGGGAGCTC-3′ (primer E). For the f39Y mutant, the internal PCR primers were oligonucleotides 5′-TCAAGAATGAAGAGGATATACTGTGATT-3′ (mutated codon in italics) and 5′-CATTGTTACCTCCTCATTACTGATT-3′. PCR reactions were carried out as described above, and PCR products were purified using the QIAquick PCR purification kit or the Qiagen Hi gel extraction kit. The restriction sites Nd6 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. CaaD 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 used to inoculate 100 mL of LB-ampicillin medium to a starting optical density at 600 nm of 0.1. After overnight growth at 30°C, cells were harvested by centrifugation (10 min at 10,000 × g), washed with 1 volume of 50 mM Tris-sulfate buffer (pH 8.2), and disrupted at 4°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 × g).

Purification of the dehalogenase. For isolation of CaaD of P. pavonaceae 170, a single colony of strain 170 was used to inoculate 100 mL of LB medium. After overnight growth at 30°C, the culture was used to inoculate 1 liter of LB medium. This culture was grown at 30°C until the early stationary-growth phase. Cells were harvested by centrifugation (10 min at 10,000 × g), washed with 1 volume of TEMAG buffer (10 mM Tris-SO4, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium azide, 10% glycerol [pH 8.0]), and stored at −20°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 CaaD expression was obtained in Pseudomonas sp. strain GJ1. A single colony of strain GJ1 containing the expression vector pDSKcaadD was used to inoculate 10 mL of LB-kanamycin medium. After overnight growth at 30°C, the culture was used to inoculate 1 liter of LB medium. This culture was grown at 30°C until the early stationary-growth phase. Cells were harvested by centrifugation (10 min at 10,000 × g), washed with 1 volume of TEMAG buffer, and stored at −20°C. CaaD was purified to homogeneity by a modification of a published procedure (47). In a typical experiment, cells of a 1-liter culture were thawed and suspended in 100 mL of TEMAG buffer. The cells were disrupted by sonication, after which broken 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 50 mM ammonium sulfate in TEMAG buffer. Fractions that showed the highest dehalogenase activity with trans-3-chloroacrylic acid were pooled and dialyzed overnight against PEMAG buffer (5 mM potassium phosphate, 1 mM EDTA, 1 mM β-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 CaaD 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 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 A1290446.

**RESULTS**

Cloning and characterization of the genes encoding CaaD. The trans-specific 3-chloroacrylic acid dehalogenase of *P. pavonaceae* sp. strain GJ1 was obtained by cloning the SaI fragment of cosmID PS41 into the SaI-linearized broad-host-range vector pDSK319, resulting in pDSKcaadD. 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 CaaD mutants were constructed using the coding sequence for the dehalogenase in plasmid pET44T2 as the template. The φP1A, φR11A, and φR11K mutants were generated by PCR using the primers 5′-CAGCGGATAATGGCGATCTTGGCGAC-3′, 5′-CAGCGGATAATGGCGATCTTGGCGAC-3′, and 5′-CAGCGGATAATGGCGATCTTGGCGAC-3′, respectively. These primers anneal to the 5′ end of the wild-type coding sequence and were used in combination with primer D. The enzyme was assayed under 1% agarose gels and PCR products were stained with ethidium bromide. Cycling conditions for the PCR 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.

PCR. PCR was carried out in a Progene DNA thermal cycler (New Brunswick Scientific Benelex B.V.). The amplification reaction mixtures (100 µL) contained standard Taq amplification buffer, 250 µM each of the four deoxyribonucleoside triphosphates, 100 ng of each primer, 100 ng of 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 PCR as described by Ho et al. (46). Two external PCR primers were oligonucleotides 5′-CACGCGGATAATGGCGATCTTGGCGAC-3′ (primer A) and 5′-TTGCGGAGGAGGGATCCCTTAGCTT-3′ (primer D). Primer A contains an NdeI restriction site (in bold) and anneals to the 5′ end of the cdaD1 gene. Primer D contains a BamHI restriction site (in bold) and anneals to the complementary sequence directly downstream of the cdaD2 gene. The internal PCR primers were oligonucleotides 5′-CATGTATCTCCTTCCAATCGCTT-3′ (primer B) and 5′-TCAAGAATACTCCGCTCTC-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 cdaD2 gene. Primer B is the complementary primer. In two separate PCRs, the AB and CD fragments were generated using cosmID PS41, 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 mixtures were subjected to electrophoresis in a 1% agarose gel, and the two PCR fragments were extracted separately using the Qiagen Hi 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 a 1% agarose gel. The restriction sites NdeI and BamHI 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 cdaD2 gene.

A dehalogenase expression vector for *Pseudomonas* sp. strain GJ1 was constructed by cloning the SaI fragment of cosmID PS41 into the SaI-linearized broad-host-range vector pDSK319, resulting in pDSKcaadD. 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.

**RESULTS**

Cloning and characterization of the genes encoding CaaD. The trans-specific 3-chloroacrylic acid dehalogenase of *P. pavonaceae* sp.
mechanism of the dehalogenase and the 4-OT homologues are shown in Fig. 1. Pairwise identities among the seven identified 4-OT homologues range from 35 to 92%.

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 tautomerasers/isomerases. Database searches identified seven related proteins as having significant sequence similarity with CaaD (Table 2). Two are well-studied enzymes involved in the bacterial catabolism of catechol to metabolites in the Krebs cycle, the 4-OT from Pseudomonas putida mt-2 (39) and the 73% identical isozyme from Pseudomonas sp. strain CF600 (41). Both are hexameric proteins that consist of identical subunits of 62 amino acid residues (4) and catalyze the isomerization of 2-oxo-4-hexene-1,6-dioate, via hydroxymuconate, to yield 2-oxo-3-hexene-1,6-dioate (51). 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 tautomerasers/isomerases.

Alignments optimizing identity between the amino acid sequences of the two dehalogenase subunits and the seven 4-OT homologues are shown in Fig. 1. Pairwise identities between the α-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. 1A. Pairwise identities between the β-subunit (CaaD2) and the 4-OT sequences are lower and range from 16 to 25% (Table 2).

The sequence alignment in Fig. 1 suggest catalytic residues for CaaD that are in the same position in the alignment as the identified catalytic residues of 4-OT. Affinity labeling (36), kinetic analysis (37), chemical synthesis (9), nuclear magnetic resonance (38, 39), site-directed mutagenesis (6), and crystal-
lographic studies (41) identified the amino-terminal proline as the catalytic base in the 4-OT-catalyzed tautomeration reaction. This N-terminal proline is invariant among all identified 4-OT homologues (Fig. 1). 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. 1), 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 (10, 41). The sequence similarity indicates that Arg-11 is present only in the α-subunit of the dehalogenase and may perform an analogous role by interacting with the carbonate group of trans-3-chloroacrylic acid.

Characterization of dehalogenase mutants. In the homohexameric (α6) 4-OT molecule, Pro-1 is important for tautomeration. 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 shown in boldface. The region of highest sequence identity among CaaD1 and the 4-OT sequences is boxed. Ppa, P. pavonaceae; Bs, B. subtilis; Psp, Pseudomonas sp.; Pp, P. putida; Ps, P. stutzeri; Sa, S. aromaticivorans; Csp, coryneform bacterial strain.

### TABLE 2. 4-OT sequences similar to CaaD

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein length (residues)</th>
<th>Organism</th>
<th>% Identity to α-Subunit</th>
<th>% Identity to β-Subunit</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylH</td>
<td>62</td>
<td>Pseudomonas putida mt-2</td>
<td>26</td>
<td>25</td>
<td>Q01468</td>
</tr>
<tr>
<td>dmpI</td>
<td>62</td>
<td>Pseudomonas sp. strain CF600</td>
<td>26</td>
<td>22</td>
<td>P49172</td>
</tr>
<tr>
<td>ywhB</td>
<td>61</td>
<td>Bacillus subtilis 168</td>
<td>35</td>
<td>16</td>
<td>CAB02512</td>
</tr>
<tr>
<td>nahl</td>
<td>62</td>
<td>Pseudomonas stutzeri AN10</td>
<td>26</td>
<td>24</td>
<td>AAD02155</td>
</tr>
<tr>
<td>xylH</td>
<td>79</td>
<td>Sphingomonas aromaticivorans F199</td>
<td>25</td>
<td>22</td>
<td>AAD03991</td>
</tr>
<tr>
<td>pnhL</td>
<td>76</td>
<td>Pseudomonas sp. strain DJ77</td>
<td>23</td>
<td>24</td>
<td>AAD03836</td>
</tr>
<tr>
<td>nahl</td>
<td>62</td>
<td>Pseudomonas 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.

### FIG. 1. Alignments of the amino acid sequences of the α-subunit (CaaD1) and β-subunit (CaaD2) of CaaD with the seven 4-OT sequences (A and B) and with the amino-terminal sequences of the subunits of the CaaD 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 shown in boldface. The region of highest sequence identity among CaaD1 and the 4-OT sequences is boxed. Ppa, P. pavonaceae; Bs, B. subtilis; Psp, Pseudomonas sp.; Pp, P. putida; Ps, P. stutzeri; Sa, S. aromaticivorans; Csp, coryneform bacterial strain.
In many reactions involving carbon-halogen bond cleavage, the carbon-halogen bond is weakened by functional groups that interact with the halogen substituent (26, 32, 49, 50). In the α-subunit of CaaD, 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 promote 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 and tyrosine. The αF39A and αF39Y mutants were still able to catalyze halide release from both dehalogenase substrates, although 5- to 10-fold slower than the wild-type enzyme (Table 3), indicating that Phe-39 is not essential for dehalogenase activity.

**DISCUSSION**

The enzyme CaaD is produced by the soil bacterium *P. pavonaceae* 170 as part of a degradative pathway for the xenobiotic nematocide *trans*-1,3-dichloropropene (27). This hydrolytic dehalogenase, of which the properties are reported in this work, has no sequence similarity with other halidohydrolases but appears to be related to the family of 4-OTs. No other bacterial 3-chloroacrylic acid dehalogenase genes have been cloned, but the N-terminal sequences of the α and β subunits of the CaaD isolated from the gram-positive coryneform bacterial strain FG41 (47) have extensive similarity with the N-terminal parts of the α and β subunits of CaaD, respectively (Fig. 1C), suggesting that these two proteins have a common evolutionary origin and are mechanistically similar. As might be expected, the two CaaD sequences are more related to each other than to the 4-OTs.

The primary amino acid sequence of 4-OT shows no apparent similarity with those of the mammalian enzymes D-dopachrome tautomerase (DDT) (42) and macrophage migration inhibitory factor (MIF) (43), nor with that of the bacterial enzyme 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) (41), but remarkably, these four proteins have a common structural architecture (24, 42). DDT, MIF, and CHMI have an almost identical subunit topology, with two ββ motifs related by pseudo-twofold symmetry and trimeric β-sheet packing (24, 41, 42). 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 twofold 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 (41–43). 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 (41, 42).

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 (6, 9, 36–39, 41). Pro-1 of MIF is required for its D-dopachrome tautomerase and phenylpyruvate tautomerase activities (22, 34). The N-terminal proline of DDT is proposed to serve as the catalytic base in the DDT-

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**TABLE 3. Activities of wild-type and mutant CaaD for *trans*-3-chloroacrylic acid (CAA) and *trans*-3-bromoacrylic acid (BAA)**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Dehalogenase sp act* (mU/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAA</td>
</tr>
<tr>
<td>Wild type</td>
<td>1,500</td>
</tr>
<tr>
<td>αP1A</td>
<td>1,260</td>
</tr>
<tr>
<td>βP1A</td>
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<tr>
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<td>110</td>
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<tr>
<td>αF39A</td>
<td>350</td>
</tr>
<tr>
<td>αF39Y</td>
<td>240</td>
</tr>
</tbody>
</table>

*Activities were measured in cell extracts containing similar amounts of dehalogenase. Halide production with 5 mM substrate was determined at 30°C and pH 8.2.*
FIG. 2. Comparative reaction scheme for 4-OT and CaaD. The primed residues come from other subunits. (A) Reaction catalyzed by 4-OT (adopted from references 10 and 41). (B) 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.
catalyzed tautomerization reaction (42). The Pro-1 residue is conserved among all known homologues of 4-OT (Fig. 1), MIF (22, 44), and DDT (42) and is also conserved in both subunits of CaaD (Fig. 1). In the CaaD isolated from strain FG41, however, the amino-terminal proline is present only in the subunit that aligns with the β-subunit of CaaD (Fig. 1C). This suggests that Pro-1 of the β-subunit may serve as a catalytic base in both of the 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 (24). The relatedness between CaaD1 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. 1A). 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 GR(S,E)K (lowercase letters indicate nonconserved positions) around the equivalent, catalytically important Arg-71 residue in CHMI. This functional motif is related to binding of the carboxylic group of the 4-OT, CHMI, and CaaD substrates.

In view of the oligomeric architecture of 4-OT, a trimer of homodimers (41), 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 (45), there are three potential active sites in CaaD. From the crystal structure of 4-OT inactivated by 2-oxo-3-pentynoate (45), 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. 2A). We propose similar roles for βPro-1 and αArg-11 in CaaD, which leads to a minimal catalytic mechanism for the CaaD-catalyzed hydrolytic dehalogenation of trans-3-chloroacrylic acid (Fig. 2B). In principle, one can write several possible chemical pathways for the CaaD-catalyzed reaction, including a nucleophilic substitution and an elimination mechanism. Although a one-step displacement of the halide by a hydroxyl ion has been suggested for the hydrolytic dehalogenation of cis- and 1,2-haloalkanoic acids by haloacid dehalogenase (DL-DEX 113) from Pseudomonas sp. strain 113 (25), 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 monofluoroformate by fumarase (23), CaaD catalyzes nucleophilic addition of a hydroxyl group on an sp²-hybridized carbon atom. This nucleophilic addition is favored 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. 2B) and the other involving the formation of a carbanion intermediate (route 2 in Fig. 2B). 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 monofluoroformate by fumarase also yielded an unstable intermediate, α-fluoroaldimine (α-fluoromalate), which subsequently decomposes to oxaloacetate and HF (23).

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 the α-subunit of CaaD, which is in the same position in the alignment as Arg-39 in 4-OT (Fig. 1), may interact with the chloride 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 (3). Phenylalanine residues were also proposed to contribute to halogen/halide binding in haloalkane dehalogenase (DhlA) and 1,2-haloacid dehalogenase (DhlB) from Xanthobacter autotrophicus GJ10 (7, 32). 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 (20, 32, 49).

Screening of the cosmids library of P. pavonaceae 170 did not reveal clones that expressed the cis-3-chloroacrylic acid dehalogenase. Thus far, 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 (47). This enzyme is probably a trimeric protein of 16,2-kDa subunits, and a comparison of its amino-terminal sequence with those of the CaaDs from strains FG41 and 170 revealed no overall similarity but showed that Pro-1 and Arg-11 are conserved (data not shown). Therefore, both cis- and trans-specific 3-chloroacrylic acid dehalogenases may catalyze the dehalogenation of their respective 3-chloroacrylic acid isomers through the mechanism shown in Fig. 2. This mechanism is different from that of most other hydrolytic dehalogenases in that it does not involve the formation of a covalent enzyme-substrate intermediate.

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