Reactions of *trans*-3-Chloroacrylic Acid Dehalogenase with Acetylene Substrates: Consequences of and Evidence for a Hydration Reaction†

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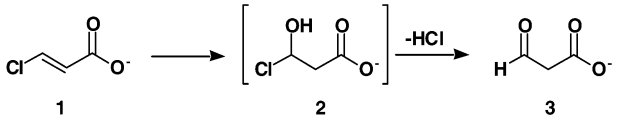
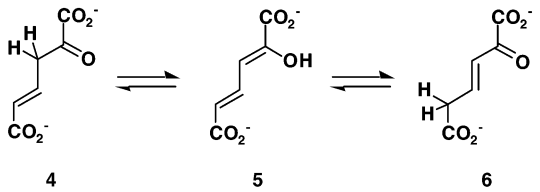
ABSTRACT: Various soil bacteria use 1,3-dichloropropene, a component of the commercially available fumigants Shell D-D and Telone II, as a sole source of carbon and energy. One enzyme involved in the catabolism of 1,3-dichloropropene is *trans*-3-chloroacrylic acid dehalogenase (CaaD), which converts the *trans*-isomers of 3-bromo- and 3-chloroacrylate to malonate semialdehyde. Sequence analysis suggested a relationship between the heterohexameric CaaD and the bacterial isomerase, 4-oxalocrotonate tautomerase (4-OT), thereby distinguishing CaaD from a number of dehalogenases whose mechanisms proceed through an alkyl- or aryl-enzyme intermediate. In this study, the genes for the α- and β-subunits of CaaD have been synthesized using a polymerase chain reaction-based strategy, cloned into separate plasmids, and the proteins expressed and purified as the functional heterohexamer. Subsequently, the product of the reaction was confirmed to be malonate semialdehyde by ^{1}H and ^{13}C NMR spectroscopy, and kinetic constants were determined using a UV spectrophotometric assay. In view of the proposed hydrolytic nature of the CaaD-catalyzed reaction, three acetylene compounds were investigated as substrates for the enzyme. One compound, 2-oxo-3-pentynoate, a potent active site-directed irreversible inhibitor of 4-OT, is a substrate for CaaD, and was processed to acetopyruvate with kinetic constants similar to those determined for the *trans*-isomers of 3-bromo- and 3-chloroacrylate. The remaining two compounds, 3-bromo- and 3-chloropropiolic acid, were transformed into potent irreversible inhibitors of CaaD. The inactivation observed for 3-bromopropiolic acid is due to the covalent modification of Pro-1 of the β-subunit. The results provide evidence for a hydratase activity and set the stage to use the 3-halopropiolic acids as ligands in inactivated CaaD complexes that can be studied by X-ray crystallography.

The enzyme *trans*-3-chloroacrylic acid dehalogenase (CaaD)[1] converts the *trans*-isomers of 3-bromo- and 3-chloroacrylates (**1**, Scheme 1) to malonate semialdehyde (**3**) (*1*−*3*). CaaD has been isolated from *Pseudomonas pavonaceae* strain 170 and is part of a pathway that enables the bacterium to use either 3-chloroacrylate or 1,3-dichloropropene as sources of carbon and energy (*4*). The latter compound is one of the active ingredients in the commercial products Shell D-D and Telone II, which are mixed in with the soil and used to control plant-parasitic nematodes (*5*).

Scheme 1



Scheme 2



The genes for the dehalogenase were recently cloned from the *P. pavonaceae* strain, the gene expressed, and the enzyme purified and characterized (*3*). The enzyme is a heterohexamer, consisting of three α-subunits, each having 75 amino acid residues, and three β-subunits, each having 70 amino acid residues. Sequence analysis revealed similarities between the α-subunit of CaaD and a bacterial isomerase, 4-oxalocrotonate tautomerase (4-OT), and the β-subunit of CaaD and a 4-OT homologue found in *Bacillus subtilis* and designated YwhB. Both 4-OT and YwhB are homohexamers, with monomers that consist of 62 and 61 amino acids, respectively (*6*, *7*).

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[1] Abbreviations: Ap, ampicillin; CaaD, *trans*-3-chloroacrylic acid dehalogenase; Cm, chloramphenicol; DMSO, dimethyl sulfoxide; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-pressure liquid chromatography; IPTG, isopropyl-β-D-thiogalactoside; Kn, kanamycin; LB, Luria-Bertani medium; MALDI-PSD, matrix assisted laser desorption-ionization post-source decay; MALDI-TOF, matrix assisted laser desorption-ionization time-of-flight; NMR, nuclear magnetic resonance; 4-OT, 4-oxalocrotonate tautomerase; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TPCK, *N*-tosylphenylalanine chloromethyl ketone.

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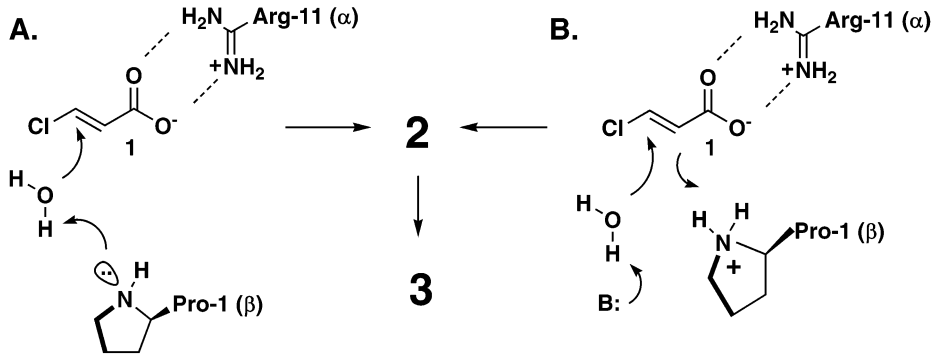
Scheme 3

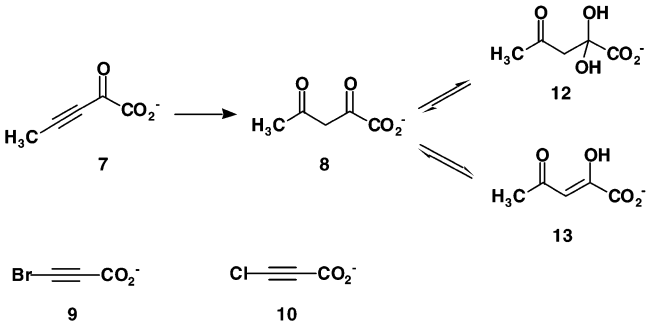


The mechanism of 4-OT has been studied extensively over the past decade (*7*). The amino-terminal proline functions as a general base, converting 2-oxo-4-hexenedioate (**4**, Scheme 2) to 2-oxo-3-hexenedioate (**6**) through a dienol intermediate, known commonly as 2-hydroxymuconate (**5**) (*7*). Arg-39 and an ordered water molecule provide hydrogen bonds to the carbonyl oxygen of **4**, thereby facilitating deprotonation at C-3 (*8*, *9*). Arg-11 interacts with the C-6 carboxylate group and functions as an electron sink to draw electron density to the C-5 position for protonation. Phe-50 plays a role in maintaining the hydrophobicity of the active site, which assists in catalysis and contributes to the lowered pK_a of Pro-1 (*10*). Significantly, both the α- and *β*-subunits of CaaD have an amino-terminal proline, while the α-subunit has Arg-11 (*3*).

These similarities and the results of mutagenesis experiments prompted Janssen and co-workers to suggest a mechanism involving the addition of water to the double bond of **1** to form the unstable chlorohydrin intermediate, **2** (*3*). Subsequent collapse to **3** can occur by either the direct expulsion of the chloride or by the α,*β*-elimination of HCl, followed by ketonization (*11*). Pro-1 of the *β*-subunit was identified as a critical mechanistic residue because the P1A mutant had no activity while mutation of Pro-1 in the α-subunit (to an alanine) had little effect on activity (*3*). Two possible roles were proposed for Pro-1 (*3*). It could function as a general base to activate the water molecule for attack at C-3 of **1** (Scheme 3A), or it could function as a general acid to protonate C-2 of **1**, while the water molecule is activated in some other manner (Scheme 3B). Arg-11 of the α-subunit was also identified as a critical residue because the R11A mutant was inactive. It is proposed that Arg-11 interacts with the C-1 carboxylate group of the 3-haloacrylate.

The mechanism of CaaD and the parallels between it and 4-OT are intriguing and raise several questions. One of the more exciting questions concerns how the two enzymes evolved and diverged to catalyze such different reactions. To gain a better mechanistic understanding of CaaD, and to ultimately address this evolutionary question, we conducted a kinetic analysis of CaaD and examined its behavior with three acetylene substrates. In the course of this investigation, we developed an efficient expression system for CaaD as well as a direct UV spectrophotometric assay to monitor its activity, and verified that **3** is the product of the reaction. We further determined that CaaD converts 2-oxo-3-pentynoate (**7**, Scheme 4) to acetopyruvate (**8**), suggesting that 3-bromo- and 3-chloropropiolioate (**9** and **10**, respectively) might function as mechanism-based inhibitors of CaaD. Both

Scheme 4



compounds were found to be potent irreversible inhibitors of CaaD with the sole target of modification by **9** being Pro-1 in the *β*-subunit. Thus, CaaD hydrated the three acetylene compounds, but the resulting products had different consequences for the enzyme. These observations are consistent with an enzyme-catalyzed hydration reaction and indicate that the 3-halopropiolic acids are promising candidates for further mechanistic and structural studies.

MATERIALS AND METHODS

Materials. All reagents, buffers, and solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), Spectrum Laboratory Products, Inc. (New Brunswick, NJ), or EM Science (Cincinnati, OH) with the following exceptions. The *trans*-3-chloroacrylate was obtained from Fluka Chemical Corp. (Milwaukee, WI). Literature procedures were used for the syntheses of 2-oxo-3-pentynoate (**7**) (*12*), 3-bromo- and 3-chloropropiolic acids (**9** and **10**), and the *trans*-isomer of 3-bromoacrylate (**11**) (*13*, *14*). Tryptone, yeast extract, and agar were obtained from Becton, Dickerson, and Company (Franklin Lakes, NJ). The Ultrafree DA centrifugal filter units, the YM-3 ultrafiltration membranes, and the Amicon concentrators were obtained from Millipore Corp. (Billerica, MA). The thin-walled PCR tubes were obtained from Ambion, Inc. (Austin, TX). Restriction enzymes, PCR reagents, and T4 DNA ligase were obtained from F. Hoffmann-La Roche, Ltd. (Basel, Switzerland), Promega Corp. (Madison, WI), or New England Biolabs (Beverly, MA). The Wizard PCR Preps DNA purification system was purchased from Promega Corp. The QIAprep Spin Miniprep kit was obtained from Qiagen, Inc. (Valencia, CA). The remaining molecular biology reagents including agarose, DNA ladders, and protein molecular weight standards, were obtained from Invitrogen Corporation

(Carlsbad, CA). Oligonucleotides for DNA amplification and sequencing were synthesized by either Oligos Etc. (Wilsonville, OR) or Genosys (The Woodlands, TX).

Strains. Escherichia coli strains DH5α (Invitrogen) and JM109 (Promega) were used for cloning and isolation of plasmids. *E. coli* strains BL21(DE3)pLysS from Novagen and BL21-Gold(DE3)pLysS from Stratagene (La Jolla, CA) were used for recombinant protein expression.

Methods. Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere (*15*), or as suggested by the manufacturer. Plasmid DNA was introduced into cells by electroporation using a Cell-Porator Electroporation System (Whatman Biometra, Göttingen, Germany). The PCR was performed in a DNA thermal cycler (model 480) obtained from PerkinElmer, Inc. (Wellesley, MA). Colony screening by the PCR is based on methods described in the pET System Manual (Novagen). DNA sequencing was done at the DNA Core Facility in the Institute for Cellular and Molecular Biology at the University of Texas (Austin). Kinetic data were obtained on a Hewlett-Packard 8452A or an Agilent 8453 Diode Array spectrophotometer. The cuvettes were mixed using a stir/add cuvette mixer (Bel-Art Products, Pequannock, NJ). The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus Software Ltd., Horley, U. K.) obtained from Sigma Chemical Co. HPLC was performed on a Waters (Milford, MA) 501/510 system or a Beckman System Gold HPLC (Fullerton, CA) using a TSKgel Phenyl-5PW hydrophobic column (TosoHaas, Montgomeryville, PA) or a Superose 12 gel filtration column (Amersham Biosciences, Piscataway, NJ). Protein was analyzed by Tris glycine sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under denaturing conditions on 17.5% gels using either the Mini-Protean II or III vertical gel electrophoresis apparatus obtained from Bio-Rad (Hercules, CA) (*16*). Protein concentrations were determined using the method of Waddell (*17*). The NMR spectra were recorded in 100% H_2O on a Varian Unity INOVA-500 spectrometer using selective presaturation of the water signal with a 2-s presaturation interval. The lock signal is dimethyl-d_6 sulfoxide. Chemical shifts are standardized to the dimethyl-d_6 sulfoxide signal at 2.49 ppm.

Construction, Overexpression, and Purification of CaaD, β-P1A-CaaD, and α-R11A-CaaD. The experimental procedures used for the synthesis of the α- and β-subunit genes of CaaD, the construction of the CaaD expression vectors, the overexpression and purification of CaaD, the construction of the β-P1A- and α-R11A-CaaD mutants, and their overexpression and purification are provided in Supporting Information.

Mass Spectrometric Characterization of CaaD and the Mutants. The subunit masses of CaaD and the two mutants were determined using an LCQ electrospray ion trap mass spectrometer (ThermoFinnigan, San Jose, CA), housed in the Analytical Instrumentation Facility Core in the College of Pharmacy at the University of Texas at Austin. The protein samples (∼1 mg/mL in 100 mM $(NH_4)_2CO_3$ buffer, pH 8.0) were mixed in a 1:10 ratio with a solution of 50% (v/v) methanol in water containing 5% acetic acid. The samples were infused into the mass spectrometer at 20 μL/min and spectra were acquired continuously in the centroid mode over the mass range of *m*/*z* 350−2000. The deconvoluted masses were calculated using ThermoFinnigan's Xcalibur 1.3 BioWorks 3.0 software with approximately 40 spectra averaged.

The observed monomer masses for the α- and β-subunits of CaaD were 8342 Da (calc. 8344 Da) and 7505 Da (calc. 7507 Da), respectively. The observed monomer masses for the α- and β-subunits of β-P1A-CaaD were 8342 Da (calc. 8344 Da) and 7479 Da (calc. 7481 Da), respectively. The observed monomer masses for the α- and β-subunits of α-R11A-CaaD were 8257 Da (calc. 8258 Da) and 7506 Da (calc. 7507 Da), respectively.

Enzymatic Assay and Kinetic Studies of CaaD. The kinetic assays were performed in 20 mM Na_2HPO_4 buffer, pH 9.0, observing the decrease in absorbance at 224 nm corresponding to the hydration of *trans*-3-chloroacrylate (**1**, $\epsilon = 4900$ M^{-1} cm^{-1}) and *trans*-3-bromoacrylate (**11**, $\epsilon = 9700$ M^{-1} cm^{-1}). An aliquot of CaaD (100 μL of a 13.2 mg/mL solution) was diluted into buffer (40 mL) and incubated for 1 h. Subsequently, a 1 mL-portion of the diluted enzyme was transferred to a cuvette and assayed by the addition of a small quantity of substrate (**1** or **11**) from a 5 or 50 mM stock solution (in 100 mM Na_2HPO_4 buffer, pH 9.2). The amount of enzyme in each 1 mL cuvette was 33 μg. The addition of **1** or **11** (as their free acids) to the 100 mM phosphate buffer adjusted the pH of the stock solution to about 7.0. The 5 mM stock solution was made by dilution of an aliquot of the 50 mM stock solution into 100 mM NaH_2PO_4 buffer, pH 7.3. The concentrations of substrate used in the assay ranged from 10 to 150 μM.

The hydration of 2-oxo-3-pentynoate (**7**) by CaaD was monitored by following the formation of acetopyruvate (**8**) at 294 nm ($\epsilon = 7000$ M^{-1} cm^{-1}) in 20 mM sodium phosphate buffer (pH 9.0) (*18*). An aliquot of CaaD (500 μL of a 2.9 mg/mL solution) was diluted into buffer (50 mL) and incubated for 1 h. The assay was initiated by the addition of a small quantity of **7** from either a 5.95 or 59.5 mM stock solution. The stock solution (59.5 mM) was made by dissolving the appropriate amount of 2-oxo-3-pentynoate in 100 mM Na_2HPO_4 buffer, pH 9.2. The addition of **7** (as the free acid) adjusted the pH of the buffer to about 7. The 5.95 mM stock solution was made by dilution of an aliquot of the 59.5 mM into 100 mM NaH_2PO_4 buffer, pH 7.3. The concentrations of **7** used in the assay ranged from 6 to 595 μM.

*^{1}H NMR Spectroscopic Detection of **3**.* An NMR tube contained 100 mM Na_2HPO_4 buffer (0.54 mL, pH ∼9.2) and a solution of **1** (4 mg, 0.04 mmol) dissolved in DMSO-d_6 (30 μL). The final pH of the solution was 6.8. Subsequently, an aliquot of CaaD (60 μL of a 6.7 mg/mL solution in 20 mM sodium phosphate buffer, pH 7.3) was added to the reaction mixture. The concentration of **1** in the NMR tube was 62 mM. The first ^{1}H NMR spectrum was recorded 5 min after the addition of the enzyme and every 5 min thereafter until the reaction was completed (30 min). **3**: ^{1}H NMR (H_2O, 500 MHz) δ 3.20 (2H, d, $J = 3$ Hz, H-2), 9.50 (1H, t, H-3). The spectra also showed signals corresponding to acetaldehyde (resulting from the nonenzymatic decarboxylation of **3**), and to the hydrates of **3** [^{1}H NMR (H_2O, 500 MHz) δ 2.34 (2H, d, $J = 5.4$ Hz, H-2), 5.15 (1H, t, H-3)] and acetaldehyde (*19*).

*^{13}C NMR Spectroscopic Detection of **3**.* An NMR tube contained 200 mM Na_2HPO_4 buffer (0.3 mL, pH ∼8.5), 1

M NaOH (175 μL), H_2O (10 μL), and a solution of **1** (20 mg, 0.19 mmol) dissolved in DMSO-d_6 (30 μL). The final pH of the solution was 6.8. After the sample sat at 4 °C overnight, an aliquot of CaaD (115 μL of a 6.7 mg/mL solution in 20 mM sodium phosphate buffer, pH 7.3) was added to the reaction mixture. The concentration of **1** in the NMR tube was 310 mM. The initial ^{13}C NMR spectrum was obtained 25 min after the addition of CaaD. **3**: ^{13}C NMR (H_2O, 125 MHz) δ 46.2 (C-2), 175.4 (C-1), 204.2 (C-3). The spectra also showed evidence for the presence of acetaldehyde (resulting from the nonenzymatic decarboxylation of **3**), and the hydrates of **3** [^{13}C NMR (H_2O, 125 MHz) δ 53.6 (C-2), 90.3 (C-3), 178.5 (C-1)] and acetaldehyde (*19*).

*UV and ^{1}H NMR Spectroscopic Detection of Acetopyruvate (**8**) in the CaaD-Catalyzed Hydration of 2-Oxo-3-pentynoate (**7**).* In the UV spectrophotometric experiment, the conversion of **7** to **8** was performed by adding a quantity of CaaD (5 μL of a 7.9 mg/mL solution) to a cuvette containing buffer (1 mL of 20 mM Na_2HPO_4 buffer, pH 9) and an aliquot of **7** (2 μL from a 59.5 mM solution in 100 mM Na_2HPO_4 buffer, pH 9.2). Spectra were recorded every 30 s until the reaction neared completion (approximately 15 min). The region from 200 to 350 nm was examined using the Agilent 8453 Diode Array spectrophotometer at 27 °C. For the ^{1}H NMR experiment, **7** (4 mg, 36 mmol) dissolved in DMSO-d_6 (30 μL), was added to 100 mM Na_2HPO_4 buffer, pH 9.2 (0.6 mL) in an NMR tube. The addition of **7** adjusted the pH of the buffer to 6.5. Subsequently, an aliquot of CaaD (200 μL of a 11 mg/mL solution) was added to the mixture to initiate the reaction. NMR spectra were recorded at 3.5 and 10 min after the addition of enzyme, when the reaction was completed. The ^{1}H NMR spectra show the presence of signals corresponding to **8**, its hydrate **12** (*20*), and the enol isomer, **13** (*20*). ^{1}H NMR (100 mM Na_2HPO_4, 500 MHz) δ 2.02 (s, 3H, CH_3 of **12**), 2.05 (s, 3H, CH_3 of **13**), 2.12 (s, 3H, CH_3 of **8**), 2.88 (s, 2H, CH_2 of **12**), 3.79 (s, 2H, CH_2 of **8**), 5.94 (s, 1H, enol proton of **13**).

Detection of the β-P1A- and α-R11A-CaaD Mutant Activity. Activities for the β-P1A- and α-R11A-CaaD mutants could not be determined by UV spectroscopy because the large amount of protein required interfered with the UV absorbance of substrate. Hence, activity was detected using ^{1}H NMR spectroscopy as follows. To an NMR tube containing 100 mM Na_2HPO_4 buffer (0.6 mL, pH ~9.2) was added a solution of 3-chloroacrylic acid (**1**, 4 mg, 0.04 mmol) dissolved in DMSO-d_6 (30 μL). The addition of **1** to the buffer lowered the pH to ~6.8. Subsequently, an aliquot of β-P1A-CaaD or α-R11A-CaaD (100 μL of a 7.4 mg/mL solution) was added to separate NMR tubes. The reaction mixture was allowed to incubate for 24 h. The final pH was 6.65 (β-P1A-CaaD) or 6.53 (α-R11A-CaaD). A ^{1}H NMR spectrum revealed the presence of acetaldehyde (resulting from the nonenzymatic decarboxylation of **3**) and its hydrate in the NMR tube containing α-R11A-CaaD, but no products in the NMR tube containing β-P1A-CaaD.

Kinetics of Irreversible Inhibition. A quantity (100 μL) of CaaD (5.7 mg/mL) was diluted 10-fold into 20 mM sodium phosphate buffer (pH 7.3) to give a final concentration of 36 μM (based on dimer molecular mass). The diluted enzyme was divided into 150 μL quantities, placed in 1.5 mL eppendorf micro test tubes, and equilibrated at 23 °C for 1 h before the addition of inhibitor. A 20 μL aliquot was removed just prior to the addition of inhibitor and assayed for activity. This activity represents 100% activity at time zero. Subsequently, varying amounts of **9** or **10** (0−40 μM) and a sufficient quantity of buffer (100 mM Na_2HPO_4 buffer, pH 7.3) were added to the incubation mixtures to make a final total volume of 140.4 μL. Aliquots (20 μL) were removed from these mixtures immediately after the addition of inhibitor (~ 5 s) and at 5-s intervals thereafter, diluted into 20 mM sodium phosphate buffer (1 mL, pH 9.0), and assayed for residual activity. The assay was initiated by the addition of **11** (150 μM). Each time point (shown in Figure 3) represents the average of three different runs. Stock solutions (50 mM) of **9** and **10** were made up in 100 mM Na_2HPO_4 buffer (pH 9.2). The addition of the inhibitor resulted in a final pH of ~7. The stock solutions were diluted with a sufficient volume of 100 mM sodium phosphate buffer (pH 7.3) to make 5 and 0.5 mM solutions of inhibitor.

*Protection of CaaD from Inhibition by **9** and **10**.* The protection of CaaD from inactivation by **9** and **10** was carried out as follows. Quantities of CaaD (150 μL) were placed in 1.5 mL eppendorf micro test tubes and the initial time point was obtained as described above. Aliquots (10.4 μL) of a solution containing either **9** (0.25 or 0.5 mM) and **11** (14, 65, and 138 mM) or **10** (0.25 or 0.5 mM) and **11** (14 mM, 65 mM, and 138 mM) were then added to the eppendorf tubes. This resulted in final concentrations of either **9** (20 or 40 μM) or **10** (20 or 40 μM) and **11** (1, 5, and 10 mM). Aliquots (20 μL) were removed from these mixtures as described above, diluted into 20 mM sodium phosphate buffer (1 mL, pH 9.0, a 50-fold dilution), and assayed for residual activity using the quantity of **11** present in the incubation mixture.

*Bromide Elimination from **9** in the Presence of CaaD.* The release of bromide ion was monitored using an Accumet AP63 pH/millivolt/ion meter equipped with an Accumet bromide combination ion selective electrode (Fisher Scientific Inc.). The amount of bromide in solution was determined from a standard curve following the manufacturer's directions. The reaction mixtures (total volume of 3 mL) contained varying amounts of **9** (0, 25, 150, or 300 μM) made up as described above and CaaD (297 μL from a 21.5 mg/mL solution resulting in a final concentration of 127 μM). The reactions were initiated by the addition of inhibitor. An aliquot (60 μL) of a 5 M $NaNO_3$ solution was added to each reaction mixture to maintain a constant ionic strength.

To determine the response time of the electrode, the amount of bromide ion was monitored in a reaction mixture containing 20 mM NaH_2PO_4 buffer, pH 9.0, (1.5 mL), $NaNO_3$ (30 μL of a 5 M solution), and NaBr (30 μL of a 5 mM solution) under the conditions described above. The final concentration of bromide ion in solution (50 μM) was determined from a standard curve to be present in solution after ~3 min.

Irreversibility of the Inactivation. The irreversibility of the reaction was established using both **9** and **10**. CaaD (1.53 μM based on the molecular weight of the dimer) was incubated with an excess of **9** or **10** (50 μM) in 5 mL of 20 mM Na_2HPO_4 buffer (pH ~ 9) for 20 min at 23 °C. The final pH of the solution was 8.0. In a separate control reaction, an identical quantity of enzyme was incubated without inhibitor under identical conditions. The treated samples had no activity after 20 min. The three samples were

dialyzed against 20 mM Na_2HPO_4 buffer, pH 9.0 at 22 °C. After 63 h, the control sample lost ~40% of its original activity, while the treated samples did not regain any activity.

Trypsin Digestion of CaaD and CaaD-Modified by ***9***. Each sample contained ~1 mg of CaaD (125 μL of a 7.9 mg/mL solution) and a sufficient quantity of 20 mM sodium phosphate buffer, pH 7.3, to give a final volume of ~0.5 mL. One sample was treated with **9** (13 μL from a 44.8 mM stock solution of **9** in 100 mM Na_2HPO_4 buffer). Both samples were incubated at 4 °C for ~12 h and passed through separate PD-10 Sephadex columns equilibrated in 100 mM $(NH_4)_2CO_3$ buffer, pH 8. Fractions (~0.5 mL) were collected, and protein was identified by the Waddell method (*17*). Subsequently, both samples were treated with sequencing grade trypsin by a modification of a literature procedure (*21*). Accordingly, aliquots (300 μL) of CaaD and modified CaaD were made 1 M in guanidine hydrochloride, 5 mM in $CaCl_2$, and 1 mM in dithiothreitol (DTT). The sequencing grade trypsin (100 μg) was reconstituted according to the manufacturer's instructions in 1 mM HCl (10 μL) and a 2-μL aliquot was added to each protein sample. The samples were incubated at 37 °C overnight. Subsequently, the mixtures were subjected to matrix assisted laser desorption−ionization (MALDI) mass spectral analysis as described below.

Mass Spectrometry of the Digested Proteins. The trypsin-digested CaaD samples were analyzed on the delayed extraction Voyager-DE PRO MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA) operating in the positive ion mode. The instrument is equipped with a 337 nm nitrogen laser with a 20 Hz firing rate. The matrix consisted of α-cyano-4-hydroxycinnamic acid from the Sequazyme Peptide Mass Standards kit (PerSeptive Biosystems) dissolved in 50% (v/v) acetonitrile in water with 0.1% trifluoroacetic acid (TFA). Samples (1 mg/mL in 100 mM $(NH_4)_2CO_3$ buffer, pH 8) were mixed with the matrix in a 1:10 ratio. A 1 μL aliquot was removed and drop dried on a stainless steel target. Close external calibration was performed using Calibration Mixture 1 (Sequazyme Peptide Mass Standards Kit) made up according to the manufacturer's directions. The 2.0 m reflector detector was used with the low mass gate set at *m*/*z* 500, and spectra were acquired over the range of *m*/*z* 500−3500. The instrument parameter file "angiotensin_reflector" was used for data acquisition, with 20 kV accelerating voltage, 74% grid voltage, 0.002% guide wire voltage and 75 ns delay time. Up to 400 shots were averaged for the spectrum with typical resolution of 8000. MALDI−Post Source Decay (PSD) spectra were composite spectra generated from a set of 11 mirror ratios acquired with the instrument parameter file "angiotensin_PSD". Up to 400 shots were averaged for each segment. Air was used for collision induced dissociation at masses less than *m*/*z* 220.

RESULTS

Construction, Expression, and Characterization of CaaD. CaaD has been constitutively expressed in both *E. coli* and *Pseudomonas* sp. strain GJ1 (*3*). To optimize expression of the unlabeled enzyme as well as isotopically labeled enzyme for NMR studies, and to facilitate mutagenesis, the genes for the α- and β-subunits of CaaD from *P. pavonaceae* strain 170 were synthesized in separate reactions using a PCR-based gene synthesis strategy (*22*−*24*), and cloned into the T7 expression system. Accordingly, the gene for the α-subunit was divided into five long oligonucleotides with short overlapping regions, while the gene for the β-subunit was divided into four long oligonucleotides with short overlapping regions. These overlaps serve as primers for the extension of the long oligonucleotides, resulting in their fusion in the course of the PCR. The full-length genes are then amplified by the inclusion of short primers (corresponding to the termini). Subsequently, the two gene fragments were cloned into separate plasmids, which were, in turn, transformed into the expression strain. The sequences of the subunits were confirmed by DNA sequencing. CaaD was purified to near homogeneity by hydrophobic interaction chromatography followed by gel filtration. Typically, the expression system yielded 17−20 mg of homogeneous protein (~95% pure as estimated by SDS−PAGE) per liter of culture.

The purified CaaD was analyzed by electrospray ionization mass spectrometry (ESI-MS) and gel filtration chromatography. A sample generates two major peaks in the mass spectrum (after deconvolution) that correspond to the expected masses of the α- and β-subunits. The observed masses of the two peaks indicate that the amino-terminal proline is not blocked in either subunit by the initiating methionine. The native molecular mass (as estimated on a gel filtration column) is comparable to the previously reported mass, suggesting that CaaD is a heterohexamer in solution (*3*).

Construction, Expression, and Characterization of CaaD Mutants. The β-P1A and the α-R11A mutants of CaaD were constructed by overlap extension PCR using the appropriate synthetic gene (in either the pET-21a or pET-24a plasmid) as template (*25*). The resulting plasmid containing the mutant gene was co-transformed into the *E. coli* strain BL21-Gold-(DE3)pLysS along with the appropriate wild-type plasmid. The sequence of each mutant was confirmed by DNA sequencing. Subsequently, the mutant proteins were expressed and purified following the protocol developed for the wild-type enzyme. The expression system yielded 10−15 mg for the β-P1A mutant and 15−20 mg for the α-R11A mutant (per liter of culture). Analysis of the mutant proteins by ESI-MS and gel filtration chromatography indicated that the α- and β-subunits of the mutants had the expected masses (and were not blocked by the initiating methionine) and that the mutants were heterohexamers in solution.

Kinetic Properties of CaaD, β-P1A-CaaD, and the α-R11A-CaaD. The activity of CaaD was previously determined by a colorimetric assay that monitored the release of the halide (*3*). It was necessary to have a more direct assay for CaaD activity in order to facilitate inhibition studies, pH rate studies, and kinetic studies of CaaD mutants. Hence, the activity of CaaD was monitored by following the decrease in the absorbance of the substrate at 224 nm. This assay was used to measure kinetic parameters for CaaD using **1** and **11** (Table 1). The two substrates have comparable K_m values, while the k_{cat} value for **11** is 1.3-fold greater than that for **1**. The increase in k_{cat} may reflect the fact that bromide is a better leaving group than chloride (if the loss of halide reflects the rate-determining step). The overall effect of the increase in k_{cat} is a slightly higher (~1.2-fold) k_{cat}/K_m value for **11** (compared to 1). A K_m of 0.19 mM and a k_{cat} of 6.4 s^{-1} were previously reported for CaaD using **1** (*3*). The

Table 1: Kinetic Parameters for CaaD[a]

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
1	3.8 ± 0.1	31 ± 2	1.2×10^5
11	5.1 ± 0.1	37 ± 2	1.4×10^5
7	0.7 ± 0.02	110 ± 4	6.4×10^3

[a] The steady-state kinetic parameters were determined in 20 mM sodium phosphate buffer (pH 9.0) at 23 °C. Errors are standard deviations.

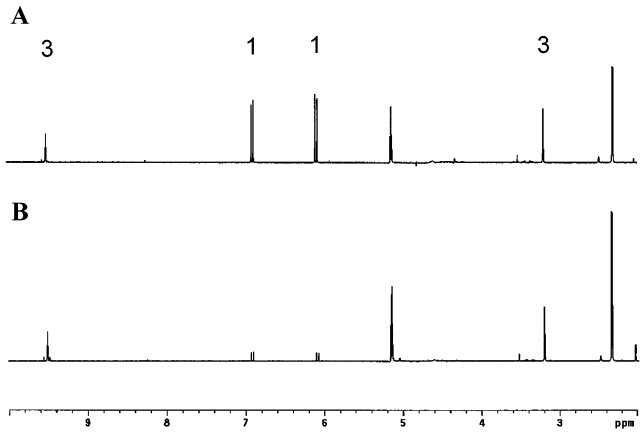


FIGURE 1: ^{1}H NMR (500-MHz, H_2O) spectra monitoring the CaaD-catalyzed conversion of **1** to **3**. (A) The initial spectrum is acquired 5 min after the addition of CaaD to a NMR tube containing **1**. The signals corresponding to protons on **1** and **3** are labeled. The signals at 2.34 and 5.15 ppm correspond to the hydrate of **3**. (B) The spectrum acquired 30 min after the addition of enzyme. The signal at 2.0 ppm corresponds to the methyl group of acetaldehyde (*19*).

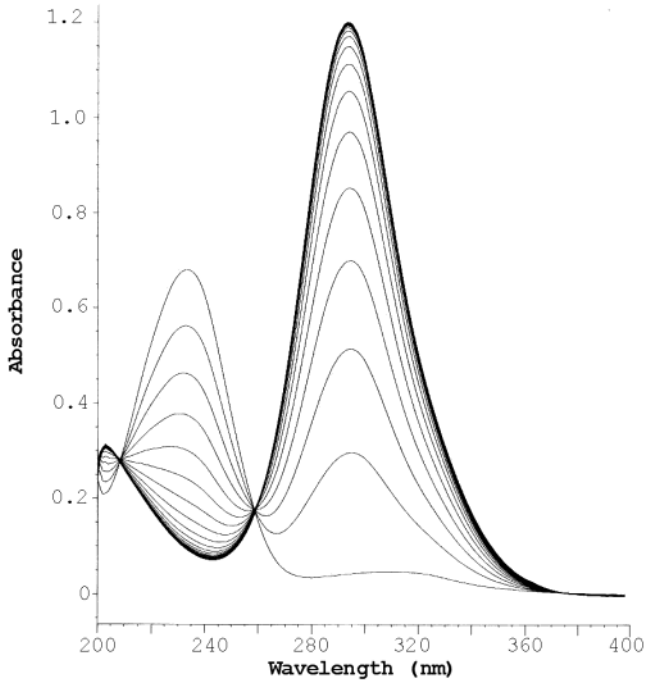


FIGURE 2: UV spectra (1-mm path length) following the CaaD-catalyzed conversion of **7** to **8**. Spectra were recorded every 30 s until the reaction neared completion (~15 min). The decrease in absorbance at 234 nm corresponds to the loss of **7** (*12*), while the production of **8** corresponds to an increase in absorbance at 294 nm (*11*).

differences (most notably in K_m) are likely a function of the two different assays.

Both the β-P1A and the α-R11A mutants of CaaD were reported to be inactive using **1** (*3*). The β-P1A mutant showed a small amount of activity using **11**, but the α-R11A mutant did not (*3*). In the spectrophotometric assay, activity was not detected for either mutant **1** or **11**. However, a 24 h incubation of both mutant proteins (in separate reactions) with **1** showed that the α-R11A mutant retained a small amount of activity (as determined by the presence of the characteristic signals in a ^{1}H NMR spectrum) while the β-P1A mutant had no detectable activity.

^{1}H and ^{13}C NMR Characterization of the CaaD-Catalyzed Reaction. To directly observe the product, the CaaD-catalyzed reaction was monitored by ^{1}H and ^{13}C NMR spectroscopy (Figure 1). Incubation of CaaD and **1** for 5 min resulted in the spectrum shown in Figure 1A. The doublets centered at 6.09 and 6.92 ppm correspond to the protons at C-2 and C-3 of **1**, respectively. Malonate semialdehyde (**3**) is responsible for the doublet at 3.20 ppm and the triplet at 9.50 ppm, which correspond to the protons at C-2 and C-3, respectively. In addition, signals corresponding to the hydrate of **3** appear as a doublet at 2.34 ppm and a triplet at 5.15 ppm, corresponding to the protons at C-2 and C-3, respectively. Finally, the spectrum shows much smaller signals readily assigned to acetaldehyde and its hydrate (*19*). After 20 min (Figure 1B), the signals corresponding to **1** have largely collapsed while those corresponding to **3** and its hydrate remain. Over time, the signals corresponding to acetaldehyde and its hydrate grow in intensity. In view of the significantly slower rate of formation of acetaldehyde, it can be reasonably concluded that it results from the nonenzymatic decarboxylation of **3**. The ^{13}C NMR spectrum (recorded 25 min after the addition of CaaD to **1**) shows signals that can be most reasonably assigned to **3** and its hydrate.

Hydration of 2-Oxo-3-pentynoate (7) by CaaD. 2-Oxo-3-pentynoate (**7**) was previously synthesized and found to be a potent active site-directed irreversible inhibitor of 4-OT (*12*). In view of the sequence similarities between CaaD and 4-OT, and the importance of the β-Pro-1 to the enzyme-catalyzed reaction (*3*), **7** was initially examined as a potential affinity label of CaaD. Incubation of **7** with CaaD resulted in a decrease in the absorbance corresponding to **7** (λ_{max} = 234 nm), accompanied by the appearance of a new absorbance peak at 294 nm, which corresponds to acetopyruvate (**8**) (Figure 2). In addition to the characteristic λ_{max}, the identity of **8** was further confirmed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum showed signals consistent with the structure of **8** (*20*), as well as two additional species, the hydrate **12** (*20*), and the enol **13** (*20*) (Scheme 4). No significant inactivation of CaaD by **7** was observed in these experiments. In the absence of CaaD, **7** is stable for several hours in solution and does not decompose to **8** (*12*).

The kinetic parameters for the conversion of **7** to **8** were also determined and are summarized in Table 1. The values of k_{cat} are 5−7-fold lower than those determined for **1** and **11**, respectively, while the K_m is about 3-fold higher. The net effect is a modest decrease (19−22-fold) in the k_{cat}/K_m (when compared with those determined for **1** and **11**). Presumably, the additional carbonyl group as well as the sp hybridization of **7** is detrimental to its binding (resulting in a higher K_m), which could misalign the substrate with respect

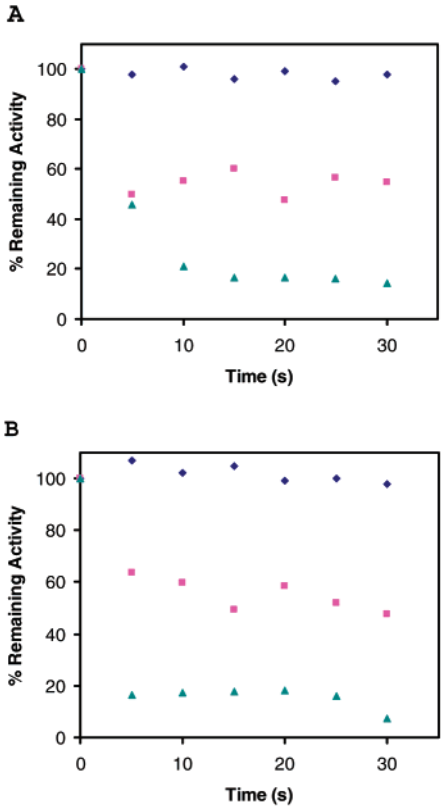


FIGURE 3: The inactivation of CaaD after incubation with varying amounts of **9** or **10** (filled diamonds, 0 μM; filled squares, 20 μM; filled triangles, 40 μM). The concentration of CaaD used is these experiments was 36 μM. The inactivation of CaaD by **9** is shown in panel A, while the inactivation of CaaD by **10** is shown in panel B.

to the catalytic groups of CaaD and cause the observed decrease in k_{cat}.

*Inactivation of CaaD by **9** or **10**.* Incubation of CaaD with either **9** or **10** results in a rapid irreversible inactivation of the enzyme (Figure 3). The enzyme loses ~80% of its activity in ~10 s when incubated with a stoichiometric excess (10% based on the dimer molecular mass of CaaD) of **9** (Figure 3A). Under the same conditions, CaaD loses 80% of its activity in ~5 s when incubated with **10** (Figure 3B). The rapid loss of activity did not permit the collection of sufficiently precise data to assess whether the loss of activity occurs in a time-dependent manner or whether the observed differences in the inactivation times are meaningful. Various attempts to slow the inactivation process by lowering the temperature to 4 °C or by varying the pH over the range of 5.5−9.0 were not successful. Under all conditions, CaaD was rapidly inactivated. Dialysis (63 h) of CaaD inactivated by either **9** or **10** does not result in any reactivation of CaaD, consistent with the formation of a covalent bond between the enzyme and a species resulting from **9** (or **10**).

Binding at the active site of CaaD was suggested by the observation that **11**, if added with either inhibitor, protects CaaD from inactivation. The results are summarized in Table 2 and show a clear trend. A lower concentration of **11** (1 mM, which is ~25-fold higher than the K_m of **11**) prolongs the enzyme's lifetime by a few seconds while higher concentrations (5 and 10 mM, which are 200−250-fold higher than the K_m of **11**) prolong it by tens of seconds.

Table 2: Protection of CaaD by **11**

[I]	1 mM **11**	5 mM **11**	10 mM **11**
20 μM **9**[a]	35% (10 s)[b]	35% (40 s)	70% (70 s)
40 μM **9**	40% (10 s)	50% (30 s)	40% (60 s)
20 μM **10**	65% (10 s)	40% (35 s)	25% (150 s)
40 μM **10**	40% (10 s)	45% (40 s)	20% (130 s)

[a] CaaD was incubated with the indicated concentrations of **9** or **10** in 20 mM sodium phosphate buffer (pH 7.3) at 23 °C along with the indicated concentrations of **11**. [b] The time interval indicates the approximate amount of activity remaining after the indicated time.

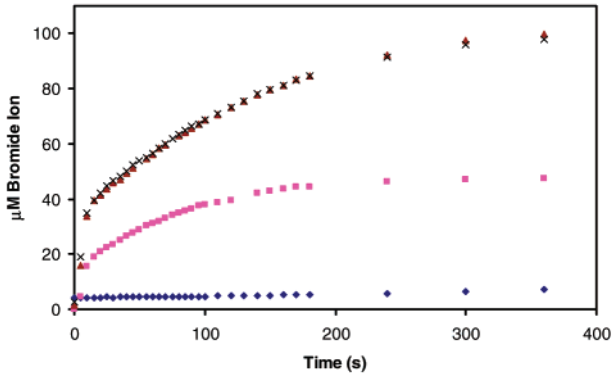


FIGURE 4: Analysis of bromide ion release from various amounts of **9** (filled diamonds, 0 μM; filled squares, 25 μM; filled triangles, 150 μM; crosses, 300 μM) upon incubation with a fixed amount of CaaD (127 μM).

Presumably, when the enzyme converts all of substrate **11** present in the incubation mixtures to **3**, the protection is lost.

*Bromide Elimination from **9** in the Presence of CaaD.* The elimination of bromide ions was observed for increasing concentrations (25, 150, and 300 μM) of **9**, when incubated with a fixed concentration of CaaD (127 μM) (Figure 4) at 24 °C and pH 8.0. In all cases, the majority of bromide ion release is complete within 3 min and is dependent on the presence of CaaD.

In the presence of a stoichiometric amount of **9**, CaaD is completely inactivated under these conditions within 10 s. This observation initially suggested that the observed bromide ion elimination and the inactivation of CaaD by **9** might not be a coupled process. However, a control experiment indicated that the rate of bromide ion release cannot be accurately determined under these conditions. In this experiment, the concentration of bromide ion in solution was monitored using a 50 μM solution of NaBr. After ~3 min, a total of 50 μM of bromide ion was achieved in solution (as detected by the electrode). Thus, the rate of CaaD inactivation by **9** is too fast to obtain accurate data that can be correlated with the rate of bromide ion elimination. Nonetheless, there is a correlation between inactivation and the release of bromide ion. At sub-stoichiometric concentrations of **9** (compared with the concentration of CaaD), bromide ion release corresponds to the concentration of **9**. When **9** is in a stoichiometric excess, bromide ion release corresponds to the concentration of CaaD.

Identification of the Modified Peptide by Mass Spectrometry. ESI mass spectral analysis of the intact α- and β-subunits of CaaD, after its incubation with **9**, indicated that only the β-subunit was modified by the inhibitor. The molecular mass of the β-subunit increases from 7505 ± 3 to 7590 ± 3 Da after incubation with **9**, while the mass of

Table 3: Mass Spectral Analysis of the Tryptic Digest of the β-Subunit

peptide fragment	calc mass (Da)[a]	obsd mass (Da) (control)	obsd mass (Da) (treated)
1−11	1200.61	1200.63	1242.68/1244.75
16−21	785.50	785.51	785.53
22−29	903.48	not detected	903.53 (weak)
30−36	703.36	not detected	703.39 (weak)
37−55	2066.11	2066.16	2066.16
56−65	1070.52	1070.54	1070.58
66−70	474.26	not detected	not detected

[a] These values were calculated using the monoisotopic MH^+ masses (Da).

the α-subunit remains unchanged at 8342 ± 3 Da. The increase in mass is due to the covalent attachment of a species with a mass of 85 ± 3 Da.

To identify the site of attachment, CaaD was inactivated by **9** (designated the treated sample), incubated overnight with trypsin, and analyzed by MALDI-TOF mass spectrometry. A control sample was made up similarly, but **9** was excluded from the mixture. The site of covalent attachment was determined by comparative MALDI-TOF of these tryptic digests followed by selected fragmentation of the peptides of interest (*26*). The results (for the β-subunit) from the mass spectral analysis of each sample are summarized in Table 3 and show one major new peak appearing in the spectrum of the treated sample.

In the MALDI spectrum of the control sample (Figure 5A), there is a prominent peak at *m*/*z* 1200.63 ± 0.06, which corresponds to the calculated mass of the N-terminal sequence (PFIECHIATGL) of the β-subunit of CaaD.[2] This peak is not present in the MALDI spectrum of the treated sample (Figure 5B), but there is a new pair of peaks at *m*/*z* 1242.68 ± 0.06 and 1244.75 ± 0.12. These masses correspond to the N-terminal sequence (PFIECHIATGL) of the β-subunit of CaaD modified by adducts of 42 and 44 Da, respectively.[3]

The ions corresponding to the selected peaks in the spectra of the control and the treated samples were subjected to MALDI−PSD analysis to determine the location of the modified residue (*26*). The MALDI−PSD spectrum of the peptide at *m*/*z* 1200 in the control sample shows a series of b ions, which have a charge on the N-terminal side of the fragmented peptide (Figure 6A). The MALDI−PSD spectra of the peptides in the treated sample show a similar b ion series, but now each ion is shifted by a mass of +42 (Figure 6B).[3] Since all the b_1 ions are shifted by +42, the modification is on the N-terminal proline. While the b_1 ion, corresponding to the fragmentation of Pro-1, is not normally seen in PSD spectra, a b_1 +42 ion is seen at *m*/*z* 140.2 in the spectra of the treated sample. In the spectrum of the intact protein, there was a mass shift of ~85 Da, while in the digest, a mass shift of +42/+44 is seen. The discrepancy is probably due to decarboxylation of the adduct during the MALDI deposition or ionization process.

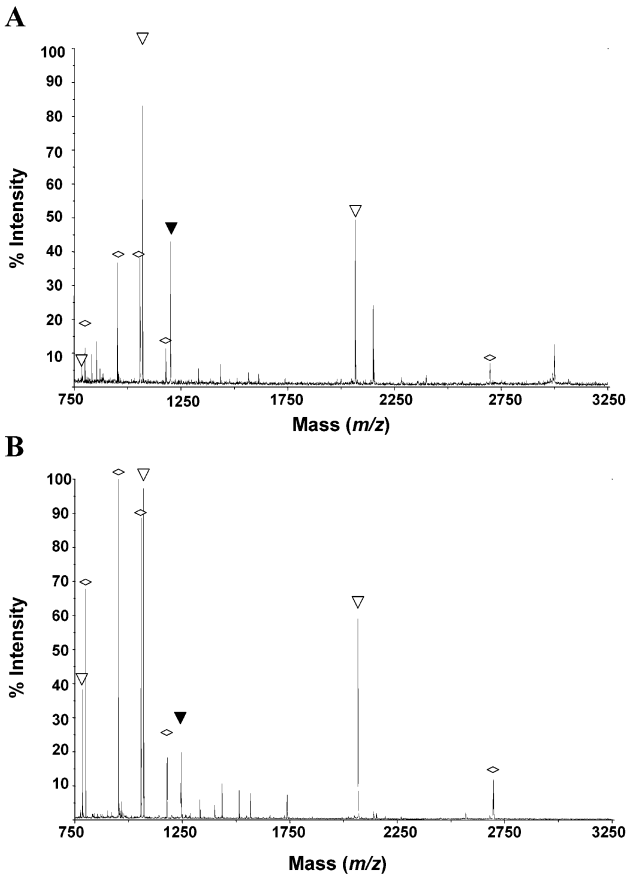


FIGURE 5: MALDI-TOF mass spectral analysis of the tryptic digest of (A) a control sample containing CaaD and (B) a CaaD sample treated with **9**. The signals corresponding to fragments of the β-subunit are labeled with open triangles, while the filled triangle designates the signal at 1200.63 ± 0.06 Da (in panel A) and at 1242.68 ± 0.06 and 1244.75 ± 0.12 Da (in panel B). The calculated and observed masses for these signals are summarized in Table 3. The signals corresponding to fragments of the α-subunit are labeled with open diamonds.

DISCUSSION

Many synthetic halogenated hydrocarbons are environmental pollutants that frequently cause adverse health effects in humans and animals (*27*). Remarkably, microorganisms have evolved to use these compounds as sources of carbon and energy, converting them to carbon dioxide and water, thereby rendering such toxic substances less harmful. In most cases, this capability stems from the presence of specialized enzymes known as dehalogenases, which catalyze the cleavage of the carbon−halogen bond. There is much interest in the mechanisms of these enzymes because of their ability to catalyze a chemically difficult reaction, their potential use in bioremediation efforts, and their possible recent origin (*27*).

The majority of known microbial hydrolytic dehalogenases can be categorized into two broad groups, based on the whether the enzyme processes a halogenated aliphatic compound (*28*, *29*) or a halogenated aromatic compound (*30*). Representative dehalogenases from both groups have been extensively studied, several crystal structures have been obtained, and a considerable body of mechanistic evidence has accumulated (*27*−*30*). These enzymes proceed through

[2] Trypsin has cleaved at the C-terminal side of Leu-11. We attribute this to the presence of chymotrypsin in the non-TPCK-treated trypsin.

[3] While the parent peptides selected for fragmentation have masses of 1242 and 1244, the b ions produced show only a mass addition of +42, instead of the expected additions of +42 and +44. One possibility is that a gas-phase rearrangement of the fragments occurred to produce only the +42 fragments.

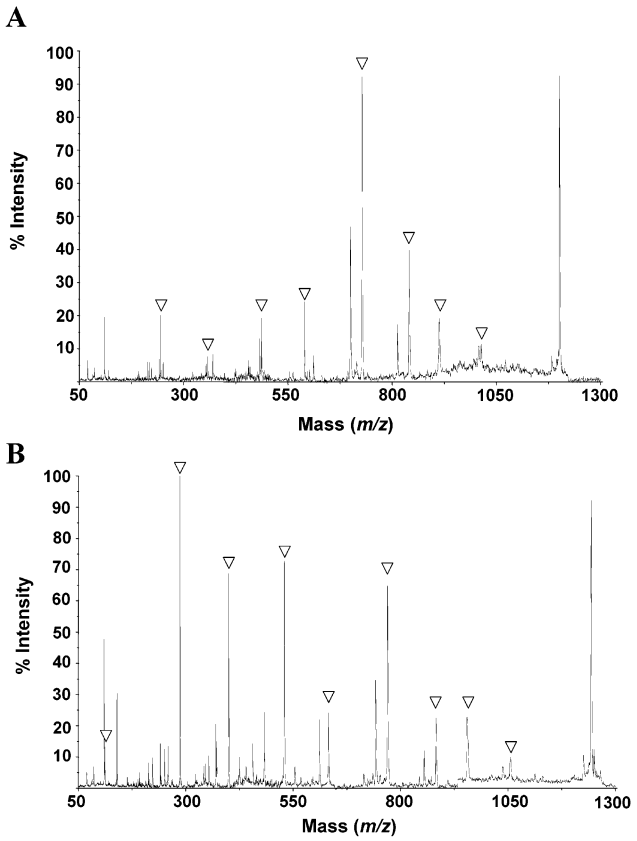


FIGURE 6: MALDI−PSD fragmentation spectra of the peaks at (A) 1200.63 Da in the control digest, corresponding to the PFIECHIATGL (1−11) fragment of the β-subunit of CaaD and at (B) 1242.68 and 1244.75 in the treated digest, corresponding to the PFIECHIATGL (1−11) modified by adducts of 42 and 44 Da, respectively. The b ions and the b_1 ions (in panels A and B, respectively) are labeled with open triangles. The b_1 ions are shifted by +42 Da.

a two-step process. In the first step, an aspartate group attacks the substrate at the carbon to which the halogen is bound, resulting in loss of the halide and the formation of an alkyl (or aryl) enzyme intermediate. Subsequently, this intermediate is hydrolyzed by water. The catalytic aspartate is part of a catalytic triad or dyad, while the departure of the halide is sometimes facilitated by the presence of a halide-binding pocket (*27*−*30*).

While haloaromatic and haloalkyl dehalogenases are well characterized, haloalkene dehalogenases are rare and the catalytic strategies used by these enzymes are poorly understood. The only known haloalkene dehalogenases are CaaD and a *cis*-3-chloroacrylic acid dehalogenase (*27*). The recent characterization of CaaD, including sequence analysis and site-directed mutagenesis studies, demonstrated clear mechanistic and structural differences between it and all previously characterized dehalogenases, and placed CaaD in the 4-OT family of enzymes (*3*).

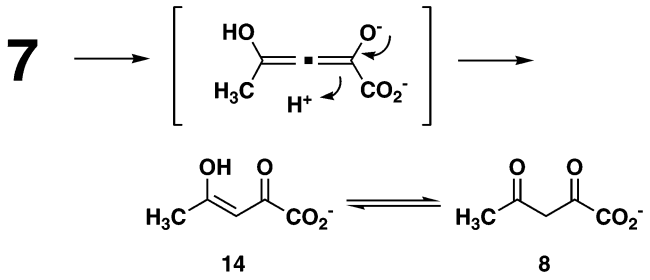
The 4-OT family of enzymes is one of the three known families that comprise the tautomerase superfamily (*31*, *32*). The other two families are represented by 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI), another bacterial isomerase, and macrophage migration inhibitory factor (MIF), a mammalian cytokine with a phenylpyruvate tautomerase activity. The members of this superfamily are homologous proteins that share two major traits: they are constructed from a common $\beta-\alpha-\beta$ structural unit and they have an amino-terminal proline. In the title enzyme of each family, Pro-1 serves as a general base in a keto−enol tautomerization reaction. In the 4-OT family, the monomer of each known member encodes the characteristic $\beta-\alpha-\beta$ unit, and these monomers form stable hexamers (4-OT and YwhB) or dimers (YdcE, a 4-OT homologue found in *E. coli*) (*32*). While the α- and β-subunits of CaaD are grouped within one of the five subfamilies in the 4-OT family, the characteristics of the subfamily are not yet well defined (*32*).

In this context, the discovery of CaaD is highly significant because it represents a new activity (hydratase) as well as a new quaternary structure (heterohexamer) in the 4-OT family. The product of the reaction, **3**, was previously identified (and quantified) by its phenylhydrazone (*1*) and formazan derivatives (*2*). Derivatization was necessary due to the facile decarboxylation of **3**. To circumvent this problem and to directly detect **3** (and other possible products), the reaction was monitored by ^{1}H NMR spectroscopy. In addition to the hydrate of **3** (which is readily stabilized by a six-member ring), the NMR spectrum showed a small amount of acetaldehyde, resulting from the facile decarboxylation of **3** (*1*, *2*). The slow rate of formation of the acetaldehyde is consistent with the nonenzymatic decay of **3**. No other products were present. On the basis of these observations, it is concluded that CaaD catalyzes primarily a hydration reaction to produce **3**, and it does not catalyze the decarboxylation of **3**.

Previous work also identified Pro-1 and Arg-11 as critical residues for the dehalogenation reaction and resulted in two proposals for the catalytic mechanism (*3*). One mechanism (Scheme 3A) involves the activation of water by Pro-1 (acting as a general base) for attack at C-3 and the subsequent protonation at C-2 by Pro-1 (now functioning as a general acid). An alternate mechanism implicates an unidentified residue in the activation of a water molecule while the proton at C-2 is derived from Pro-1, functioning only as a general acid (Scheme 3B). In both mechanisms, Arg-11 plays a role in binding and perhaps catalysis: it interacts with the carboxylate group of the substrate and may function as an electron sink to facilitate catalysis. Electron density is drawn away from C-3 by the interaction with Arg-11, which generates a partial positive charge.

Whether Pro-1 functions as a general base or acid catalyst, our mutagenesis results further reinforce the critical nature of Pro-1 in the CaaD-catalyzed reaction. It was not possible to quantify the enzymatic activity of the β-P1A mutant (as well as the α-R11A mutant) using a more sensitive UV assay, which measures the depletion of substrate at 224 nm. However, it was determined by NMR spectroscopy that incubation of **1** and the β-P1A mutant for 24 h did not produce acetaldehyde, which would result from the nonenzymatic decay of **3**. In 24 h, the α-R11A mutant generated a small quantity of acetaldehyde, suggesting, in turn, that a small amount of **3** had formed. In the absence of a crystal structure, it is not clear whether the inactivity of the β-P1A mutant and the diminished activity of the α-R11A are due to structural perturbations in addition to the absence of essential catalytic groups. Interestingly, the R11A mutation of 4-OT is significantly more detrimental to catalytic activity than is the P1A mutation, primarily due to an 8.6-fold increase in K_m (*8*, *33*). The mutations did not result in major structural perturbations to the 4-OT structure (*8*, *33*).

Scheme 5



It is noteworthy that the reaction catalyzed by CaaD is a particularly difficult one, as has been reported for other enzyme-catalyzed dehalogenation reactions. In 0.5 M aqueous NaOH at 60 °C, about 10% of the chloride is removed from **1** in 24 h (*34*). The kinetic analysis indicates that CaaD accelerates this process considerably and that **11** is a slightly better substrate for CaaD than is **1**. The basis for this difference in substrate reactivity is not readily determined. While the C-3 position of **1** is more susceptible to nucleophilic attack by water due to the greater activation at this position by the chloride, bromide is a better leaving group (*35*). Hence, it is necessary to identify the rate-determining step in order to discern whether one of these factors is involved.

CaaD will also process three acetylene compounds to products, which have different consequences for the enzyme. The first compound, 2-oxo-3-pentynoate (**7**), an irreversible inhibitor of 4-OT, functions as a substrate for CaaD, and is converted to acetopyruvate, which does not inactivate the enzyme. The most reasonable scenario for the formation of **8** from **7** is shown in Scheme 5. The CaaD-catalyzed addition of water to **7** results in the initial formation of the allenol species, which is protonated to afford the enol species, **14**. Subsequent ketonization of **14** produces **8**. It has previously been demonstrated that **8**, in aqueous solution, will form both the hydrate, **12**, and the enol, **13** (*20*), accounting for the presence of these products in the spectrum. Interestingly, **7** is a reasonably good substrate for CaaD with a k_{cat}/K_m value nearing that measured for **1** and **11**.

The observation that **7** is a substrate and not an inhibitor reflects the difference between the active sites of CaaD and 4-OT. CaaD is set up to do a hydration reaction while 4-OT carries out an isomerization reaction. As a result, the active site of CaaD is likely to be more hydrophilic in nature than the active site of 4-OT. Pro-1 of 4-OT is able to function as a general base at physiological pH because its pK_a is lowered to about 6.4. The hydrophobic active site of 4-OT plays a major role in lowering the pK_a of Pro-1 and in providing a favorable environment for catalysis (*10*).

The other two acetylene compounds, **9** and **10**, are also substrates for the enzyme, but upon hydration they are converted into potent inhibitors of CaaD, with **9** being shown to modify Pro-1 of the β-subunit. Modification of β-Pro-1 can occur by a mechanism-based route to form an acyl halide (or a ketene) or by Michael addition of β-Pro-1 to C-3 (Scheme 6). In the first mechanism (Scheme 6A), the enzyme hydrates **9** (or **10**) to generate an unstable species **15**, which will readily undergo tautomerization to generate the acyl halide (**16**), or elimination to produce the ketene (**17**). Due to their reactivity, both species would be lethal to the enzyme.

In the second mechanism (Scheme 6B), Pro-1 attacks the C-3 position of **9** in a Michael-type reaction. While conjugate additions to α,β-unsaturated carboxylic acids are not normally known, the inhibitor would presumably be activated by an interaction between the carboxylate group of **9** and a residue on the enzyme (i.e., α-Arg-11). Subsequent rearrangement would result in the covalent attachment of the propargyl moiety to the enzyme and the loss of the halide ion.

The observation that the covalent adduct on Pro-1 has a molecular mass of 85 Da is consistent with the attachment of a 3-oxoacetate moiety. Moreover, this adduct, a β-keto-acid, is quite susceptible to facile decarboxylation upon covalent attachment, resulting in an acetyl group (*36*). At first glance, this sequence of events seems consistent with the mechanism-based route. However, the 3-oxoacetate moiety could also be derived from a rearrangement of the propargyl moiety as shown in Scheme 7. A nonenzymatic rearrangement of the propargyl moiety would result in the formation of an electrophilic allene-type compound (**18**), which would be highly vulnerable to attack by a water molecule. Ketonization of the resulting enol could generate the observed 3-oxoacetate moiety.

The detection of the acyl halide, the ketene, or their hydrolysis product, malonate, would provide a key piece of evidence supporting the mechanism-based route to inactivation. Thus far, they have remained elusive and are likely to remain so. If either species is generated by the enzyme, it must immediately alkylate the enzyme as there is no evidence for its release into solution. This premise is supported the observation that the inactivation of CaaD and the bromide ion release upon inactivation are both nearly stiochiometric. If immediate alkylation did not occur, then CaaD would continue to process **9**, and the concentrations of bromide ion release would be greater than the concentrations of enzyme.

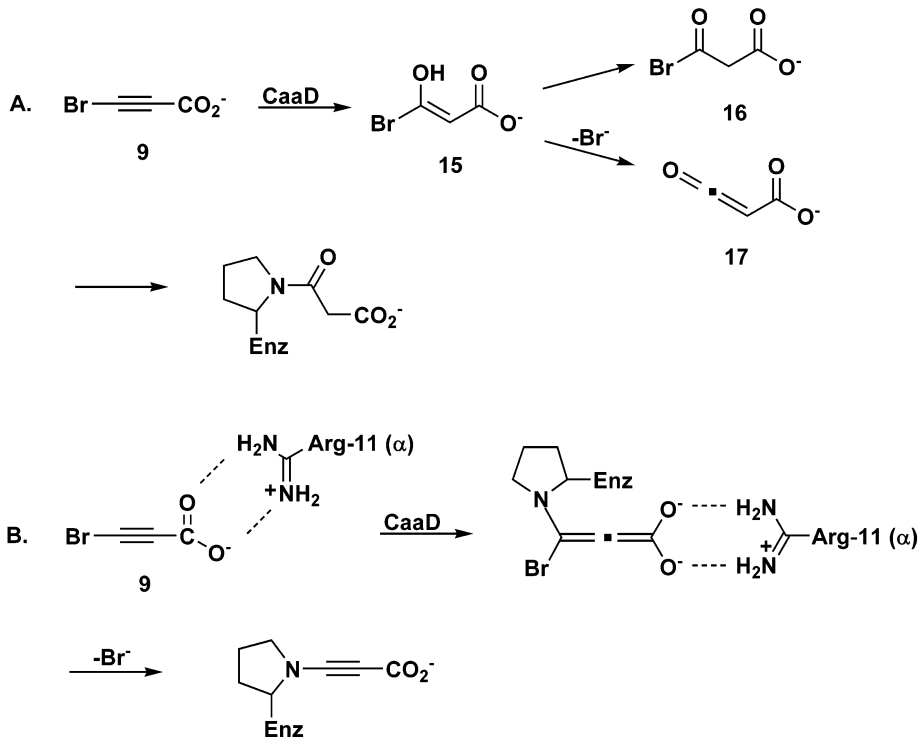
While our current data are consistent with either route, the mechanism-based route is favored by the observation that **7** is converted to acetopyruvate (**8**) and recent experiments indicating that β-Pro-1 has a pK_a of $\sim$9.2,[4] making a significant portion of the enzyme not sufficiently nucleophilic to add to **9** or **10** in a Michael type reaction.[5] Interestingly, if Pro-1 functions as a general acid catalyst in the reaction, the initial hydration of **9** (or **10**) by CaaD would remove a proton from Pro-1 and render it nucleophilic and quite susceptible to acylation by the acyl halide or reaction with the ketene. Further studies are being actively pursued to discriminate between these mechanisms and a crystal structure of the inactivated enzyme will soon be available.

Our studies with the three acetylene compounds raise an intriguing possibility about the biological function of CaaD as well as its evolution. The CaaD-catalyzed processing of the acetylene compounds is consistent with a hydratase reaction, suggesting that this might be the true physiological role of CaaD in the organism (or perhaps the original role). The loss of the halide from **1** or **11**, after the addition of water, might be a fortuitous side reaction resulting from the

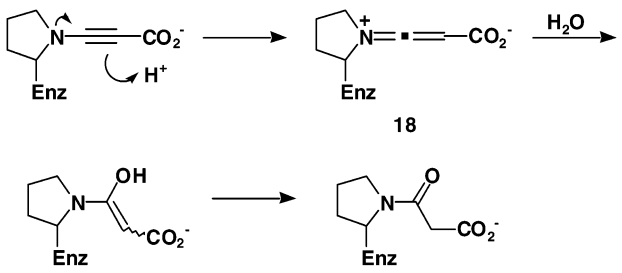
[4] Azurmendi, H. F., Wang, S. C., Massiah, M. A., Whitman, C. P., Mildvan, A. S. (2003), unpublished results.

[5] The inactivation studies were carried out at pH 9.0. From the pK_a value of 9.2, it can be estimated that at pH 9.0, 61% of the enzyme is not in the correct protonation form to function as a general base.

Scheme 6



Scheme 7



very rapid nonenzymatic decay of the unstable halohydrin. This would make CaaD an "accidental" dehalogenase (*27*), which might have been conscripted to serve in the catabolic pathway for 1,3-dichloropropene. This speculation is further supported by the observation that CaaD does not appear to have a halide-binding pocket (*3*). Identification of the putative physiological substrate for CaaD would further assist in the delineation of its evolution.

Whatever the original function of CaaD is in the organism, the relationships between its α- and *β*-subunits and 4-OT and YwhB are interesting and raise questions about their evolutionary lineage. In one possible scenario, an ancestral chromosomally encoded enzyme, having a nonspecific tautomerase activity as well as other low-level activities, could give rise to the 4-OT activity and the CaaD activity through a duplication event followed by a series of mutations to obtain the desired activity (*37*, *38*). Both 4-OT and CaaD retain the ability to carry out a common chemical step, which is the protonation of the carbon adjacent to the carboxylate group, involving Pro-1 and Arg-11. In 4-OT, the Arg-11 functions as an electron sink to draw electron density toward C-5 for protonation by Pro-1. In CaaD, Arg-11 may also function as an electron sink to draw electron density away from C-3 (resulting in a partial positive charge at C-3) and thereby facilitate protonation at C-2 by Pro-1. While the physiological function of YwhB is not yet known, we have recently determined that both 4-OT and YwhB have low-level CaaD activities, which may result from the presence of this catalytic core (*7*). These observations reinforce the hypothesis that the *β*−α−*β* structural motif is a versatile template (*7*), which nature has clearly exploited to create the new activity and structure displayed by CaaD.

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SUPPORTING INFORMATION AVAILABLE

Experimental procedures used for the synthesis of the α- and *β*-subunit genes of CaaD, the construction of the CaaD expression vectors, the overexpression and purification of CaaD, the construction of the *β*-P1A- and α-R11A-CaaD mutants, and their overexpression and purification. This material is available free of charge via the Internet at http://pubs.acs.org.

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