Stereospecific Alkylation of *cis*-3-Chloroacrylic Acid Dehalogenase by (*R*)-Oxirane-2-carboxylate: Analysis and Mechanistic Implications†

Gerrit J. Poelarends, Hector Serrano, William H. Johnson, Jr., and Christian P. Whitman*

Division of Medicinal Chemistry, College of Pharmacy, The University of Texas, Austin, Texas 78712-1074

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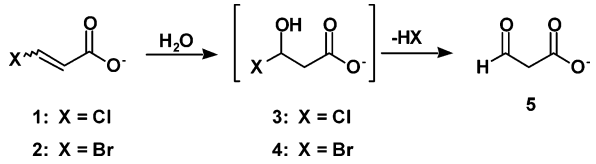
ABSTRACT: The enzymes *trans*-3-chloroacrylic acid dehalogenase (CaaD) and *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD) represent the two major classes of bacterial, isomer-selective 3-chloroacrylic acid dehalogenases. They catalyze the hydrolytic dehalogenation of either *trans*- or *cis*-3-haloacrylates to yield malonate semialdehyde, presumably through unstable halohydrin intermediates. In view of a proposed general acid/base mechanism for these enzymes, (*R*)- and (*S*)-oxirane-2-carboxylate were investigated as potential irreversible inhibitors. Only *cis*-CaaD is irreversibly inhibited in a time- and concentration-dependent manner and only by the (*R*)-enantiomer of oxirane-2-carboxylate. The enzyme displays saturation kinetics and is protected from inactivation by the presence of substrate. These findings indicate that the inactivation process involves the initial formation of a reversibly bound enzyme−inhibitor complex at the active site followed by covalent modification. Mass spectral analysis of the inactivated *cis*-CaaD shows that Pro-1 is the site of modification. It has also been determined that Arg-70 and Arg-73 are required for covalent modification because incubation of either the R70A or R73A mutant with inhibitor does not result in enzyme alkylation. Studies of the pH dependence of the kinetic parameters of wild-type *cis*-CaaD reveal that a protonated group with a pK_a of ∼9.3 is essential for catalysis. The group is likely Pro-1, making it predominately a charged species under the conditions of the inactivation experiments. Two mechanisms could account for these observations. In one mechanism, the oxirane undergoes acid-catalyzed ring opening followed by alkylation of the conjugate base of Pro-1. Alternatively, the oxirane undergoes a nucleophilic substitution reaction where the conjugate base of Pro-1 functions as the nucleophile and an acid catalyst polarizes the carbon oxygen bond. The two arginine residues likely bind the carboxylate group and position the inhibitor in a favorable orientation for the alkylation reaction. These findings set the stage for a crystallographic analysis of the inactived enzyme to delineate further the roles of active site residues in both the inactivation process and the catalytic mechanism.

3-Chloroacrylic acid dehalogenases are hydrolytic dehalogenases that convert 3-haloacrylates (**1** and **2** in Scheme 1) to malonate semialdehyde (**5**), presumably through unstable halohydrin intermediates (**3** and **4**) (*1*−*3*). These enzymes are found in several soil bacteria where they are involved in the metabolism of the xenobiotic nematocide 1,3-dichloropropene or the related degradation products 3-chloroallyl alcohol and 3-chloroacrylic acid (ref *4* and references cited therein). There are two major classes of 3-chloroacrylic acid dehalogenases with different isomer selectivities, *trans*-3-chloroacrylic acid dehalogenases and *cis*-3-chloroacrylic acid dehalogenases. Recently, the genes encoding the *trans*-3-chloroacrylic acid dehalogenase (CaaD)[1] from the 1,3-dichloropropene-degrading bacterium *Pseudomonas pavonaceae* 170 and the gene encoding the *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD) from the 3-chloroacrylic acid-degrading coryneform bacterium strain FG41 have been cloned and expressed, the enzymes purified, and the key elements of the mechanisms determined (*1*−*3*, *5*, *6*).

Scheme 1



The 3-chloroacrylic acid dehalogenases are unique because they cleave a vinylic carbon−halogen bond without a cofactor requirement. They belong to the tautomerase superfamily, a group of structurally homologous proteins characterized by a conserved $\beta-\alpha-\beta$ structural motif and a catalytically important amino-terminal proline (*1*, *3*). The heterohexameric CaaD consists of relatively small α-subunits (75 amino acids) and β-subunits (70 amino acids) (*1*, *5*). The mechanism has been explored by site-directed mutagenesis (*1*, *2*, *5*, *6*), irreversible inhibitors (*2*), pH−rate profiles (*6*), NMR spectroscopy (*6*), and X-ray crystallography (*5*).

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* To whom correspondence should be addressed. Tel: 512-471-6198. Fax: 512-232-2606. E-mail: whitman@mail.utexas.edu.

[1] Abbreviations: CaaD, *trans*-3-chloroacrylic acid dehalogenase; *cis*-CaaD, *cis*-3-chloroacrylic acid dehalogenase; CHMI, 5-(carboxymethyl)-2-hydroxymuconate isomerase; DMSO, dimethyl sulfoxide; ESI-MS, electrospray ionization mass spectrometry; MALDI-PSD, matrix-assisted laser desorption/ionization post-source decay; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MSAD, malonate semialdehyde decarboxylase; MIF, macrophage migration inhibitory factor; 4-OT, 4-oxalocrotonate tautomerase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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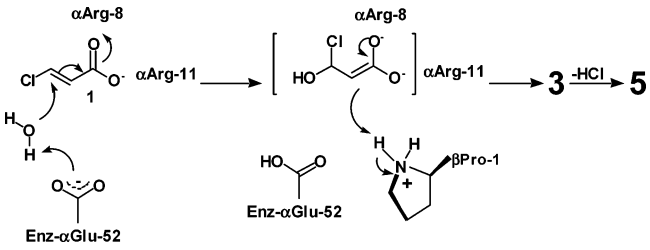
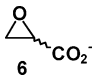
Scheme 2



On the basis of these experiments, it has been proposed that αGlu-52 acts as the general base catalyst to activate a water molecule for attack at C-3 (of *trans*-**1** or *trans*-**2**) and that *β*Pro-1 functions as the general acid catalyst to provide a proton at the C-2 position (Scheme 2). Two arginines, αArg-8 and αArg-11, likely interact with the carboxylate group to align the substrate and draw electron density away from C-3. Such an interaction would facilitate the Michael addition of water to generate **3** (or **4**). The enzymatic or nonenzymatic collapse of **3** (or **4**) produces **5**.

In contrast, *cis*-CaaD functions as a homotrimer, where each monomer is composed of 149 amino acids (*3*). Although a crystal structure is not yet available for the enzyme, sequence analysis and site-directed mutagenesis studies suggest a mechanism for *cis*-CaaD, which largely parallels that of CaaD (*3*). Accordingly, Glu-114 functions as the water-activating base while Pro-1 places a proton at the C-2 position (of *cis*-**1**). Two arginine residues (Arg-70 and Arg-73) interact with the carboxylate group of the substrate, which would assist the presumed Michael addition of water. There are also mechanistic differences between the two enzymes. One difference involves the activation of the water molecule. For *cis*-CaaD, this process probably involves additional residues because the k_{cat}/K_m value determined for the E114Q mutant is only 8-fold less than that measured for wild type (*3*). In CaaD, αGlu-52 appears to be largely responsible for the activation of water because the αE52Q mutant is inactive (*5*).

In an effort to identify additional active site residues as well as to delineate more fully the differences between CaaD and *cis*-CaaD, the (*R*)- and (*S*)-enantiomers of oxirane-2-carboxylate (**6**) were investigated as potential irreversible



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inhibitors. Only *cis*-CaaD was irreversibly inhibited by the (*R*)-enantiomer of **6**, which covalently modifies Pro-1. Further characterization of the inactivation process demonstrated that Arg-70 and Arg-73 are necessary for alkylation of Pro-1, but Glu-114 is not. Interestingly, Pro-1 is a charged species at the pH used in the inactivation experiments. This supposition is based on the observation that the pH−rate profiles for the kinetic parameters of *cis*-CaaD show a pK_a value of 9.3 ± 0.2 for a protonated group on the free enzyme, which can be reasonably assigned to Pro-1. Hence, inactivation of *cis*-CaaD may proceed by the acid-catalyzed ring opening of the oxirane followed by alkylation of the conjugate base of Pro-1 or by a nucleophilic substitution reaction involving the conjugate base of Pro-1. The two arginine residues could position the inhibitor in a favorable orientation for the alkylation reaction by interacting with the carboxylate group. The results provide a foundation for future crystallographic studies of the inactivated *cis*-CaaD complex to shed light on the structural basis for the stereospecific alkylation of this enzyme by (*R*)-oxirane-2-carboxylate.

MATERIALS AND METHODS

Materials. Chemicals, biochemicals, buffers, and solvents were purchased from Fisher Scientific Inc. (Pittsburgh, PA), Fluka Chemical Corp. (Milwaukee, WI), or Sigma-Aldrich Chemical Co. (St. Louis, MO), unless stated otherwise. Literature procedures were used for the synthesis of the racemic mixture of oxirane-2-carboxylate (**6**), as well as the (*R*)- and (*S*)-enantiomers (*7*). Tryptone, yeast extract, and agar were obtained from Becton, Dickerson, and Co. (Franklin Lakes, NJ). The Amicon concentrater and YM10 ultrafiltration membranes were obtained from Millipore Corp. (Bedford, MA). The membrane tubing was purchased from Spectrum Laboratory Products, Inc. (Gardena, CA). Prepacked PD-10 Sephadex G-25 columns were purchased from Biosciences AB (Uppsala, Sweden). Endoproteinase Glu-C (protease V-8) and protein molecular weight standards were obtained from F. Hoffmann-La Roche, Ltd. (Basel, Switzerland). CaaD, *cis*-CaaD, and the *cis*-CaaD mutants were purified to homogeneity, as assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), according to published procedures (*2*, *3*).

General Methods. For the purification of the enzymes, HPLC was performed on a Waters (Milford, MA) 501/510 system using either a TSKgel DEAE-5PW (150 × 21.5 mm) or a TSKgel Phenyl-5PW (150 × 21.5 mm) column (Tosoh Bioscience, Montgomeryville, PA). Protein was analyzed by SDS−PAGE under denaturing conditions on gels containing 15% polyacrylamide (*8*). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined by the method of Waddell (*9*). Absorbance data were obtained on a Hewlett-Packard 8452A 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. Nuclear magnetic resonance (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 (DMSO-d_6). Chemical shifts are standardized to the DMSO-d_6 signal at 2.49 ppm.

pH Dependence of the Kinetic Parameters of cis-CaaD. The pH dependence of the steady-state kinetic parameters was measured in 20 mM sodium phosphate buffers with pH values ranging from 6 to 10 (*6*). For each pH value, a sufficient quantity of enzyme was equilibrated in buffer (20 mL) for ∼1 h at 23 °C. The addition of a large amount of enzyme changed the pH, so that the reported pH values are those determined after the addition of enzyme. Subsequently, aliquots (1 mL) were removed and assayed for activity using *cis*-**1** concentrations ranging from 1 to 200 *μ*M. Stock solutions of *cis*-**1** were made up as described (*3*). To show that the decrease in activity at high pH (>9.0) was due to the enzymatic reaction, rather than the result of denaturation, the enzyme was equilibrated at 23 °C in 20 mM Na_2HPO_4

buffer (pH 10) for 1 h. An aliquot (100 μL) was then diluted into 20 mM Na_2HPO_4 buffer (pH 9.0) and immediately assayed with 150 μM of *cis*-**1**. The resulting activity was comparable to that expected for the enzyme at pH 9.0, indicating that *cis*-CaaD was not irreversibly denatured at the high pH values investigated.

*Examination of (R,S)-, (R)-, and (S)-**6** as Irreversible Inhibitors of CaaD and cis-CaaD*. CaaD and *cis*-CaaD (~1 mg/mL, ~20 μM in monomer concentration) were incubated separately with (*R,S*)-, (*R*)-, or (*S*)-**6** (2 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3). The mixtures (total volume of 100 μL) were incubated for ~24 h at 4 °C. Aliquots (4 μL) of these mixtures were then withdrawn, diluted into 1 mL of 20 mM Na_2HPO_4 buffer (pH 9.0), and assayed for residual activity. CaaD and *cis*-CaaD activity were monitored by following the decrease in absorbance at 224 nm, which corresponds to the hydration of *trans*-**1** ($\epsilon = 4900\ M^{-1}\ cm^{-1}$) or *cis*-**1** ($\epsilon = 2900\ M^{-1}\ cm^{-1}$) (*2*, *3*). The activity assay was initiated by the addition of a small quantity of the appropriate isomer of **1** to give a final concentration of 100 μM. Both enzymes are saturated under these conditions. Stock solutions (50 mM) of *trans*- and *cis*-**1** were made up in 100 mM Na_2HPO_4 buffer, and the pH was adjusted to 7.3. Stocks solutions (100 mM) of the inhibitors were made up in 100 mM NaH_2PO_4 buffer (pH 7.3). The inactivated enzymes were dialyzed for 24 h at 4 °C with 20 mM NaH_2PO_4 buffer (pH 7.3), and activity was measured as described above.

*Kinetics of Irreversible Inhibition of cis-CaaD by (R,S)- and (R)-**6***. The time-dependent inactivation of *cis*-CaaD by (*R,S*)- and (*R*)-**6** was determined by the incubation of varying amounts of inhibitor (0−60 mM) with enzyme (15 μM) in 20 mM NaH_2PO_4 buffer (pH 7.3) at 22 °C. The incubation mixtures (total volume of 100 μL) were made up in 1.5 mL Eppendorf micro-test tubes. Aliquots (5 μL) from these mixtures were removed at various time intervals, diluted into 1 mL of 20 mM Na_2HPO_4 buffer (pH 9.0), and assayed for residual activity as described above. The observed rate constant for inactivation (k_{obs}) at each inhibitor concentration was determined from a nonlinear least-squares fit of the data for loss in enzymatic activity as a function of incubation time to the equation for a first-order decay. At all concentrations of inactivator used, the decrease in activity was pseudo first order in enzymatic activity for at least three half-lives. The k_{obs} values for inactivation were plotted against the initial inhibitor concentrations, and the kinetic parameters (K_I and k_{inact}) were determined by fitting these data to a rectangular hyperbola by nonlinear least-squares analysis as previously described (*10*, *11*).

*Protection from Inactivation of cis-CaaD by (R,S)-**6***. Protection against the inactivation of *cis*-CaaD by (*R,S*)-**6** was carried out as described above with the following modifications. The enzyme (15 μM) was incubated with varying concentrations of *cis*-**1** (0−10 mM) in 50 mM NaH_2PO_4 buffer (pH 7.3) at 22 °C. After a 30 s interval, a fixed concentration of (*R,S*)-**6** (2 mM) was added to the mixture. Aliquots (5 μL) were removed at various time intervals, diluted into 1 mL of 20 mM Na_2HPO_4 buffer (pH 9.0), and assayed for residual activity as described above.

Active Site Titration of cis-CaaD. A series of samples was prepared by mixing the appropriate amount of (*R*)-**6** with *cis*-CaaD in 20 mM NaH_2PO_4 buffer (pH 7.3) to vary the [I]/[E] ratio between 1 and 500, where [E] refers to the monomer concentration of enzyme. The mixtures were incubated at 4 °C for 72 h. Aliquots (5 μL) of the reaction mixtures were then withdrawn, diluted into 1 mL of 20 mM Na_2HPO_4 buffer (pH 9.0), and assayed for residual activity as described above. The residual activity was plotted versus the molar ratio of (*R*)-**6** to *cis*-CaaD (expressed as the monomer concentration).

*Mass Spectral Analysis of (R,S)-**6**-Treated cis-CaaD and Mutants*. In these experiments, *cis*-CaaD and the P1A, R70A, R73A, and E114Q mutants were treated with (*R,S*)-**6** in separate incubation mixtures as follows. Each sample contained ~1 mg of enzyme (100 μL of a 10 mg/mL solution) and a sufficient quantity of 20 mM NaH_2PO_4 buffer (pH 7.3) to give a final volume of 0.5 mL. Each sample was treated with (*R,S*)-**6** [5 μL from a 100 mM stock solution of (*R,S*)-**6** in 100 mM NaH_2PO_4 buffer, pH 7.3] and incubated at 4 °C for ~24 h. In a separate control experiment, the same quantity of *cis*-CaaD was incubated without inhibitor under otherwise identical conditions. Subsequently, the incubation mixtures were loaded onto separate PD-10 Sephadex G-25 gel filtration columns, which had previously been equilibrated with 100 mM NH_4HCO_3 buffer (pH 8.0) (*2*, *3*, *11*). The proteins were eluted by gravity flow using the same buffer. Fractions (0.5 mL) were analyzed for the presence of protein by UV absorbance at 214 nm. The appropriate fractions containing the purified proteins were analyzed by electrospray ionization mass spectrometry (ESI-MS). In addition, the two purified *cis*-CaaD samples [untreated and treated with (*R,S*)-**6**] were assayed for residual activity and were used in the peptide mapping experiments described below.

The masses of untreated *cis*-CaaD, *cis*-CaaD treated with (*R,S*)-**6**, and the four mutants of *cis*-CaaD treated with (*R,S*)-**6** 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 were made up as described elsewhere (*2*).

Peptide Mapping. A quantity (~27 μg) of unmodified *cis*-CaaD or *cis*-CaaD modified by (*R,S*)-**6** in 27 μL of 100 mM NH_4HCO_3 buffer (pH 8.0) was combined with 3 μL of 10 M guanidine hydrochloride and incubated for 1 h at 37 °C. Subsequently, the protein samples were incubated for 48 h at 37 °C with sequencing grade protease V-8 (2 μL of a 10 mg/mL stock solution made up in water) (*12*). These protease V-8 treated samples were made up and analyzed on the delayed extraction Voyager-DE PRO matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) instrument (PerSeptive Biosystems, Framingham, MA) as described previously (*2*, *3*). Selected ions in the samples were also subjected to MALDI-post-source decay (PSD) analysis using the protocol described elsewhere (*2*, *3*).

*Kinetics of Reversible Inhibition of cis-CaaD and CaaD by (R)- and (S)-**6***. The reversible inhibition of CaaD was examined using (*R*)- and (*S*)-**6**, while the reversible inhibition of *cis*-CaaD was examined using (*S*)-**6**. For each experiment, a small amount of enzyme was diluted into 15 mL of assay buffer [20 mM NaH_2PO_4 buffer (pH 7.3)] to give a final enzyme concentration of 1 μM (CaaD) or 0.2 μM (*cis*-CaaD) (in oligomer). Subsequently, an aliquot of inhibitor from a stock solution [1 M in 100 mM NaH_2PO_4 buffer (pH 7.3)] was added to the diluted enzyme solution to yield the approximate final inhibitor concentration. After 1 h, aliquots

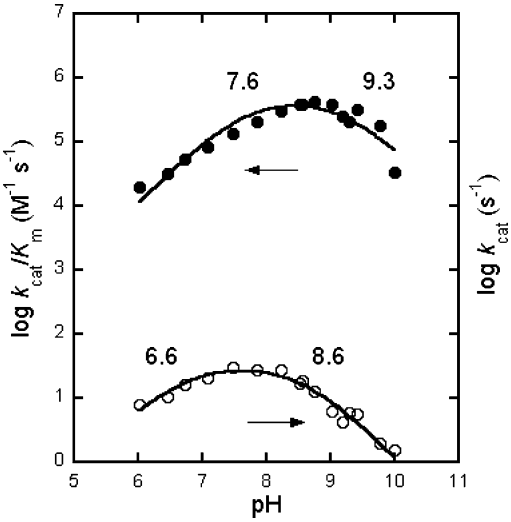

FIGURE 1: pH dependence of log k_{cat}/K_m (●) and log k_{cat} (○) for the hydration of *cis*-**1** by *cis*-CaaD. The curves were generated by a nonlinear least-squares fit of the data to either eq 1 or eq 2 as discussed in the text.

(1 mL) of the resulting solution were removed and assayed using 12 concentrations (from 10 to 175 μM) of the appropriate isomer of **1**. The final concentrations of the inhibitors ranged from 0 to 10 mM. The mode of inhibition was determined from Lineweaver−Burk reciprocal plots (*13*). The inhibition constants (K_I values) were obtained by fitting the data by nonlinear regression data analysis using the equation for competitive inhibition provided by the Grafit program.

*Examination of (R)- and (S)-**6** as Substrates for CaaD and cis-CaaD.* ^{1}H NMR spectra monitoring the reaction between CaaD and (*R*)- and (*S*)-**6** and that between *cis*-CaaD and (*S*)-**6** were recorded at 23 °C as follows. An amount of inhibitor (4 mg) dissolved in DMSO-d_6 (30 μL) was added to 100 mM Na_2HPO_4 buffer (0.6 mL, pH 9.2) and placed in an NMR tube. The pH of the mixture was adjusted to 7.3. Subsequently, an aliquot of the enzyme (0.3 mg in 20 mM NaH_2PO_4 buffer, pH 7.3) was added to the reaction mixture. In a separate control reaction, the same amount of (*R*,*S*)-**6** was incubated without enzyme under otherwise identical conditions. A ^{1}H NMR spectrum of each reaction mixture was recorded 24 h after the addition of enzyme.

RESULTS

pH Dependence of the Kinetic Parameters of cis-CaaD. The pH dependences of k_{cat} and k_{cat}/K_m for *cis*-CaaD using *cis*-**1** were studied over the pH range 6−10. Both parameters show a bell-shaped dependence on pH (Figure 1), with limiting slopes of unity on either side of the pH maximum, indicating that both a basic and an acidic group on the enzyme are important for catalysis where only the single protonated form of the enzyme is active. The pH dependences of k_{cat} and k_{cat}/K_m are given by the equations:

$$k_{cat}(\mathrm{pH}) = k_{cat}/(1 + [H^+]/K_1 + K_2/[H^+]) \quad (1)$$

$$k_{cat}/K_m(\mathrm{pH}) = k_{cat}/K_m/(1 + [H^+]/K_1 + K_2/[H^+]) \quad (2)$$

where K_1 is the ionization constant of the basic group, K_2 is the ionization constant of the acidic group, k_{cat} is the k_{cat} of the enzyme in the single protonated form, and k_{cat}/K_m is the k_{cat}/K_m of the enzyme in the single protonated form (*6*, *14*).

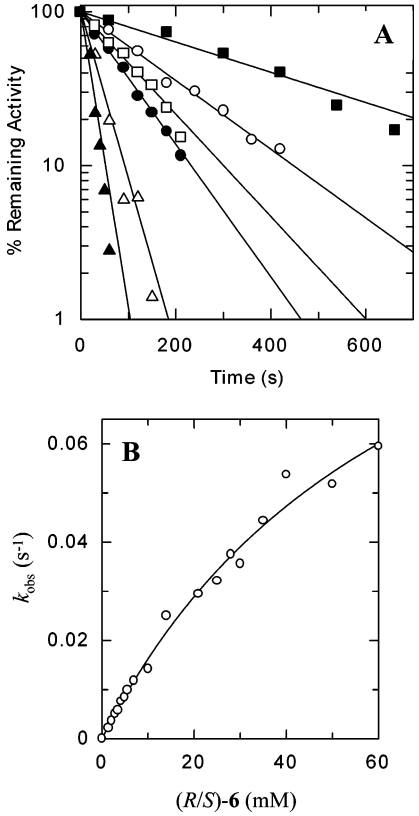

FIGURE 2: Time- and concentration-dependent irreversible inactivation of *cis*-CaaD by (*R*,*S*)-**6**. (A) A logarithmic plot showing the percent of *cis*-CaaD activity remaining as a function of the incubation time with varying amounts of (*R*,*S*)-**6** (filled rectangles, 1.4 mM; open circles, 2.8 mM; open rectangles, 4.2 mM; filled circles, 5.6 mM; open triangles, 14 mM; filled triangles, 35 mM). (B) Plot of k_{obs} as a function of (*R*,*S*)-**6** concentration. The data from this plot were used to calculate k_{inact} and K_I, which are reported in the text.

A fit of the pH dependence of k_{cat} (Figure 1), which follows the ionization of the enzyme−substrate complex, to eq 1 yields pK_a values for the enzyme−substrate complex of pK_1 = 6.6 ± 0.1 and pK_2 = 8.6 ± 0.1. A fit of the pH dependence of k_{cat}/K_m (Figure 1), which follows the ionization of the free enzyme, to eq 2 gives pK_a values for the free enzyme of pK_1 = 7.6 ± 0.2 and pK_2 = 9.3 ± 0.2. These values can be assigned to the free enzyme because the substrate has no ionizable groups in the pH range 6−10 (*6*).

*Irreversible Inactivation of cis-CaaD by (R,S)-**6**.* To determine whether oxirane-2-carboxylate (**6**) irreversibly inhibits CaaD or *cis*-CaaD, the enzymes were incubated in separate reaction mixtures with a racemic mixture of **6**. After 24 h, CaaD was not significantly inhibited by a 100-fold excess of (*R*,*S*)-**6**. However, *cis*-CaaD had no residual activity after being incubated for 24 h with a 100-fold excess of (*R*,*S*)-**6**. Exhaustive dialysis and gel filtration do not result in significant reactivation of the *cis*-CaaD inhibited by (*R*,*S*)-**6**, suggesting that a covalent bond has formed between the oxirane and the enzyme.

*Kinetic Characterization of cis-CaaD Inactivation by (R,S)-**6**.* The inactivation of *cis*-CaaD by (*R*,*S*)-**6** occurs in a time- and concentration-dependent manner (Figure 2A). Plots of the logarithm of the residual activity versus time were

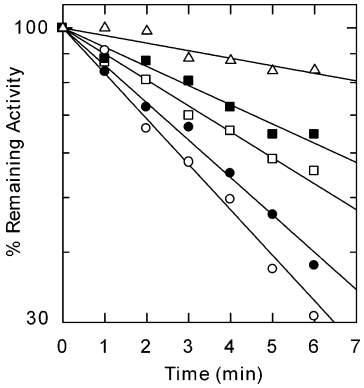

FIGURE 3: Protection of *cis*-CaaD against inactivation by (*R*,*S*)-**6** using the substrate *cis*-**1**. *cis*-CaaD was incubated with varying amounts of *cis*-**1** (open circles, 0 mM; filled circles, 1 mM; open rectangles, 3 mM; filled rectangles, 5 mM; open triangles, 10 mM) for 30 s before the addition of (*R*,*S*)-**6** (2 mM).

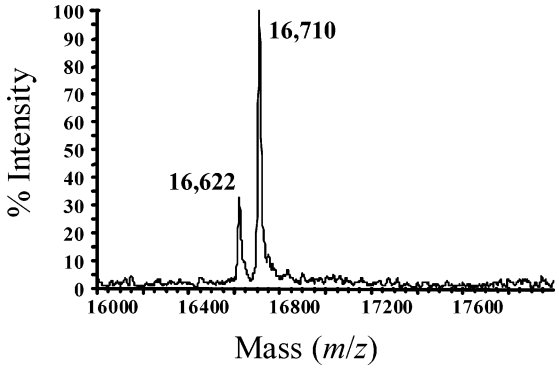

FIGURE 4: ESI-MS spectrum of *cis*-CaaD modified by **6**. The deconvoluted spectrum displays a single charged state. The major component in the spectrum is the modified *cis*-CaaD (*m*/*z* 16710), and the minor component is the unmodified *cis*-CaaD (*m*/*z* 16622).

linear, indicating that the rate of inactivation follows pseudo-first-order kinetics (*15*). The k_{obs} values measured in 18 experiments were plotted versus the initial inhibitor concentrations and fit to a rectangular hyperbola (Figure 2B). From this plot, the maximal rate of inactivation (k_{inact}) and the apparent dissociation constant for the reversibly formed enzyme−inhibitor complex (K_I) were estimated to be 0.13 ± 0.01 s^{-1} and 70 ± 10 mM, respectively. The observed saturation kinetics suggests that a dissociable complex forms between the enzyme and (*R*,*S*)-**6** at the active site before covalent bond formation and inactivation (*15*). Binding at the active site is further indicated by the observation that the substrate, *cis*-**1**, protects the enzyme against inactivation by (*R*,*S*)-**6** (Figure 3) (*15*).

ESI-MS Analysis of the cis-CaaD Modified by (R,S)-6. To obtain further evidence for the covalent modification of *cis*-CaaD by (*R*,*S*)-**6** and to ascertain whether single or multiple modifications had occurred, the (*R*,*S*)-**6**-inactivated protein was subjected to ESI-MS analysis and the spectrum (Figure 4) compared to that obtained for the native *cis*-CaaD. The spectrum resulting from ESI-MS analysis of the untreated *cis*-CaaD shows a single peak corresponding to a mass of 16622 ± 2 Da (data not shown). The spectrum of *cis*-CaaD inactivated by (*R*,*S*)-**6** shows a minor signal at 16622 ± 2 Da, which corresponds to unmodified enzyme, and a major signal at 16710 ± 2 Da, which corresponds to the modified *cis*-CaaD. Hence, incubation of *cis*-CaaD with (*R*,*S*)-**6** results in an increase in the mass of the protein due to the covalent attachment of a species with a mass of 88 Da. This mass corresponds to the expected molecular mass of **6**, as its ring-opened derivative, indicating that only one molecule of **6** is covalently bound to a *cis*-CaaD subunit.

Table 1: Identification of Peptides Produced by the Protease V-8 Digestion of *cis*-CaaD and *cis*-CaaD Modified by **6**

peptide fragment	calcd mass[a]	obsd mass	
		cis-CaaD	**6**-modified *cis*-CaaD
^{1}P−^{26}D	2860.5	2860.5	2948.5
^{62}T−^{71}E	1208.7	not detected	1208.6
^{72}G−^{94}E	2398.3	2398.3	2399.2
^{95}I−^{106}E	1562.8	1562.8	1562.8
^{115}Y−^{125}E	1300.6	1300.6	1300.6
^{137}R−^{141}E	653.3	653.3	653.3
^{142}T−^{149}T	891.5	not detected	891.5

[a] The monoisotopic singly charged masses are predicted from analysis of the translated amino acid sequence of the *cis-caaD* gene (corresponding to *cis*-CaaD).

ESI-MS Analysis of cis-CaaD Mutants Treated with (R,S)-6. To determine the role of the putative active site residues Pro-1, Arg-70, Arg-73, and Glu-114 in the alkylation reaction, the *cis*-CaaD mutants P1A, R70A, R73A, and E114Q were incubated in individual reaction mixtures with (*R*,*S*)-**6** for 24 h at 4 °C. Subsequently, the (*R*,*S*)-**6**-treated protein samples were purified by gel filtration chromatography and analyzed by ESI-MS. Mass spectral analysis of the (*R*,*S*)-**6**-treated samples of P1A, R70A, and R73A showed that each consists of one major component with observed molecular masses of 16596 ± 2, 16536 ± 2, and 16536 ± 2 Da, respectively (data not shown). These masses correspond to the expected molecular masses of the unmodified mutants. The observation that the P1A, R70A, and R73A mutants cannot be labeled by **6** indicates that Pro-1, Arg-70, and Arg-73 are essential for the alkylation reaction. Mass spectral analysis of the (*R*,*S*)-**6**-treated sample of the E114Q mutant indicated that it consists of one major component with an observed molecular mass of 16709 ± 2 Da, which corresponds to the expected molecular mass of the E114Q mutant covalently modified by **6**. Thus, in contrast to Pro-1, Arg-70, and Arg-73, Glu-114 is not important for the alkylation reaction.

Identification of the Modified Residue by Mass Spectrometry. Incubation of *cis*-CaaD with (*R*,*S*)-**6** leads to the covalent attachment of a species with a mass of 88 Da to the enzyme (vide supra). To identify the site of attachment, samples of *cis*-CaaD and *cis*-CaaD modified by **6** were digested with endoproteinase Glu-C (protease V-8), and the resulting peptide mixtures were analyzed by MALDI-MS. At pH 8.0, in 100 mM ammonium bicarbonate buffer, protease V-8 cleaves peptide bonds at the carboxylate side of glutamate and aspartate residues, with a preference for the former (*12*).

Mass spectral analysis of the two peptide mixtures indicated that proteolytic cleavage occurred predominantly at Asp-26, Asp-61, Glu-71, Glu-94, Glu-106, Glu-114, Glu-125, Glu-136, and Glu-141 (Table 1). The MALDI-MS spectrum of the modified *cis*-CaaD sample revealed the presence of two species with masses of 2860.5 and 2948.5 Da (Figure 5). The minor species (2860.5 Da) corresponds to the mass of the Pro-1 to Asp-26 peptide fragment. The major species (2948.5 Da) corresponds to the same peptide

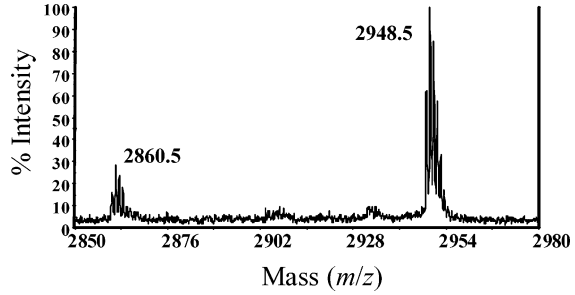

FIGURE 5: MALDI-MS spectrum displaying the protease V-8 digested *cis*-CaaD after incubation with **6**. The spectrum displays a major species with an observed mass of 2948.5 (single charged state) that corresponds to the mass of the 26-residue amino-terminal peptide plus the covalent addition of 88 Da as well as a minor component (2860.5), which corresponds to the unmodified 26-residue amino-terminal peptide.

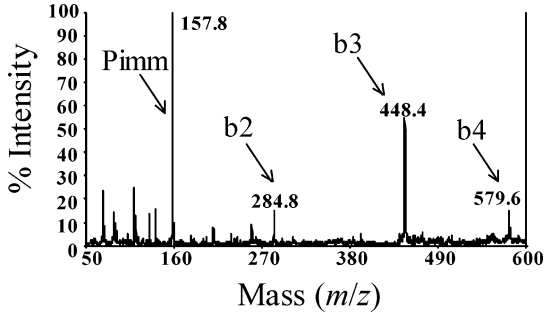

FIGURE 6: The MALDI-PSD spectrum of the precursor ion *m*/*z* 2948.5 obtained with peptide Pro-1−Asp-26, which has been modified by **6**. The fragment ions b_2 (*m*/*z* 284.8), b_3 (*m*/*z* 448.4), and b_4 (*m*/*z* 579.6) correspond respectively to the PV, PVY, and PVYM fragments modified by a species with a mass of 88 Da. The immonium ion (*m*/*z* 157.8) corresponds to the fragmentation of Pro-1, modified by a species with a mass of 88 Da.

fragment modified by **6**. The MALDI-MS spectrum of the unmodified *cis*-CaaD displays only the species with a mass of 2860.5 Da (data not shown). Examination of the remaining peaks showed that no other peptide fragments had been modified (Table 1).

To identify the site of covalent modification, the modified and unmodified peptides were subjected to MALDI-PSD analysis (*16*). The PSD spectrum of the ion (*m*/*z* 2860.5) corresponding to the unlabeled peptide (Pro-1 to Asp-26) displayed the N-terminal sequence-specific fragment ions b_2, b_3, b_4, and b_5 (data not shown). PSD analysis of the ion (*m*/*z* 2948.5) corresponding to the modified peptide fragment revealed an increase in mass of 88 Da of the b_2, b_3, and b_4 fragment ions (Figure 6). Because the b_2 ion results from the fragment Pro-1 to Val-2, only these residues remain as potential targets of alkylation. Further evidence implicating Pro-1 as the site of modification was provided by the presence of the characteristic immonium ion resulting from Pro-1 in the PSD spectrum of the modified peptide, with a mass value (i.e., 157.8 Da) consistent with the covalent attachment of a single molecule of **6** to Pro-1.

Inactivation of cis-CaaD by (R)-6 and Kinetic Characterization. The observed irreversible inhibition of *cis*-CaaD by the racemic mixture of **6** raised the question of whether one enantiomer was solely responsible for inactivation or whether both enantiomers were functioning as inactivators, although with different potencies. To address this issue, the individual enantiomers of **6** were synthesized and incubated

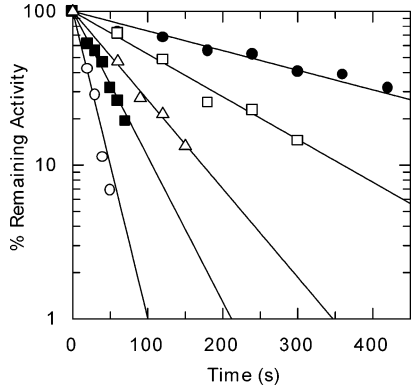

FIGURE 7: Time- and concentration-dependent irreversible inactivation of *cis*-CaaD by (*R*)-**6**. A logarithmic plot of the percent of remaining *cis*-CaaD activity as a function of the incubation time using varying concentrations of (*R*)-**6** (filled circles, 1 mM; open squares, 2 mM; open triangles, 4 mM; filled squares, 8 mM; open circles, 14 mM).

with the enzyme. After a 24 h incubation period with a 100-fold excess of (*R*)-**6**, *cis*-CaaD had no residual activity. Incubation of *cis*-CaaD with the (*S*)-enantiomer under the same conditions had no effect on activity. Hence, (*R*)-**6** is solely responsible for the irreversible inhibition of *cis*-CaaD.[2]

The inactivation of *cis*-CaaD by (*R*)-**6** also occurs in a time- and concentration-dependent manner (*15*). A logarithmic plot showing the percent of *cis*-CaaD activity remaining as a function of time using different concentrations of (*R*)-**6** is shown in Figure 7. At all concentrations of (*R*)-**6** used, the decrease in activity was pseudo first order for at least three half-lives. A plot of the k_{obs} values obtained from 12 experiments versus inhibitor concentration (1−14 mM) gave a straight line through the origin indicative of nonsaturation kinetics (data not shown). Our inability to observe saturation kinetics stems from the rapid inactivation at the higher inhibitor concentrations (>14 mM). At these concentrations, the rapid rate of inactivation precluded the collection of sufficiently precise data to obtain k_{obs} values.

To determine the stoichiometry of the inactivation reaction, the residual enzymatic activity was measured as a function of the total equivalents of (*R*)-**6** used (Figure 8). The activity decreases to about 28% with a 1.7-fold excess of (*R*)-**6** (over the concentration of active sites). By extrapolating to 0% activity, it can be estimated that only a small excess (∼2.5-fold) of (*R*)-**6** is required to achieve full enzyme inactivation.

Reversible Inhibition of CaaD and cis-CaaD. Although (*S*)-**6** does not inactivate *cis*-CaaD, it slows the rate of inactivation of *cis*-CaaD by the (*R*)-enantiomer. Therefore, (*S*)-**6** was examined as a potential reversible inhibitor of *cis*-CaaD. A Lineweaver−Burk plot using three different inhibitor concentrations shows that (*S*)-**6** is a competitive inhibitor with a K_I value of 9.2 ± 0.8 mM. In addition, both the (*R*)- and (*S*)-enantiomers were found to be competitive inhibitors of CaaD, with K_I values of 3.5 ± 0.4 and 6.6 ± 0.9 mM, respectively. These results show that both enantiomers of **6** bind at the active sites of both CaaD and *cis*-CaaD and suggest that only the (*R*)-enantiomer is positioned in a

[2] The enantiomers of **6** were synthesized from (*R*)- and (*S*)-serine, which have enantiomeric excesses of >97% (according to the manufacturer). Hence, it is estimated that (*R*)- and (*S*)-**6** have comparable enantiomeric excesses.

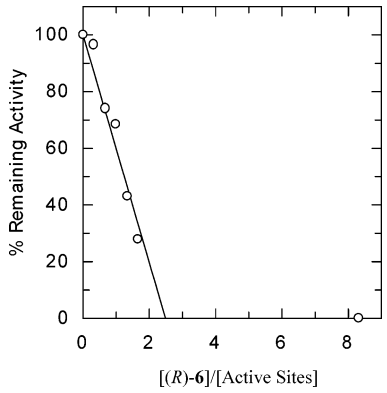

FIGURE 8: Effect of (*R*)-**6** on the catalytic activity of *cis*-CaaD. A plot showing the percentage of residual activity versus the molar ratio of (*R*)-**6** to enzyme active sites. At a ratio of 8.3 and higher, the enzyme has no detectable activity.

favorable orientation in the active site of *cis*-CaaD for an alkylation reaction to occur.

Examination of (R)- and (S)-6 as Potential Substrates of CaaD and cis-CaaD. One possible reason for the inability of (*S*)-**6** to inactivate *cis*-CaaD or for either enantiomer of **6** to inactivate CaaD is that these compounds are substrates for the enzymes. The enzyme-catalyzed hydrolysis of **6** would result in ring opening and the formation of a diol product. To explore this possibility, CaaD was incubated with both enantiomers of **6** (in separate reactions) while *cis*-CaaD was incubated with (*S*)-**6**, and the reactions were monitored by ^{1}H NMR spectroscopy. After incubation of the reaction mixtures for 24 h at 23 °C, the ^{1}H NMR spectra showed no evidence for the presence of the diol products, indicating that the epoxides are not substrates for CaaD or *cis*-CaaD. Moreover, the enzymes do not lose significant activity during the incubation period, which is consistent with preceding results showing that the two enzymes are not inactivated by these oxiranes.

DISCUSSION

Catalytic Mechanism of cis-CaaD. The recent characterization of CaaD and *cis*-CaaD, which represent the two major classes of isomer-specific 3-chloroacrylic acid dehalogenases, suggested that they share a conserved catalytic mechanism despite their different primary and quaternary structures, isomer specificity, and catalytic efficiencies (*1*−*3*). Sequence analysis also indicated that CaaD and *cis*-CaaD belong to separate families within the tautomerase superfamily, which suggests an independent evolution of the mechanisms (*1*, *3*). These observations provoked several questions about the structural and evolutionary relationship between CaaD and *cis*-CaaD, their evolutionary origins, the structural basis for the isomer specificity, and the relationship between these dehalogenases and the other tautomerase superfamily members.

A working hypothesis for the catalytic mechanism of *cis*-CaaD has been formulated using sequence analysis and the known three-dimensional structures of CaaD (*5*) and 4-oxalocrotonate tautomerase (4-OT), the best characterized enzyme of the tautomerase superfamily (*3*, *17*). This analysis implicated Pro-1, Arg-70, Arg-73, and Glu-114 as important active site residues. Subsequent mutagenesis confirmed that these residues are all required for optimal *cis*-CaaD activity (*3*). On the basis of these observations, it has been postulated that *cis*-CaaD carries out a hydrolytic dehalogenation of *cis*-**1** (or *cis*-**2**) using a general acid/base mechanism where Glu-114 functions as the general base to activate water for the addition to C-3 of the substrate and Pro-1 acts as a general acid to protonate C-2 (*3*). The Michael addition of water to the C-3 position is facilitated by the interaction of the two arginine residues with the oxygens of the C-1 carboxylate group. This interaction stabilizes a putative enediolate intermediate, which would result in a partial positive charge at C-3. Ketonization of the enediolate and protonation at C-2 by Pro-1 would complete the addition of water and generate the unstable halohydrin. Collapse of the halohydrin, either a nonenzymatic or an enzyme-catalyzed process, results in **5**.[3]

pH Dependence of the cis-CaaD-Catalyzed Reaction and Mechanistic Implications. The pH dependence of the kinetic parameters for the *cis*-CaaD-catalyzed reaction is comparable to that observed for the CaaD-catalyzed reaction and provides support for the proposed general acid/base mechanism. The pH−rate profile for *cis*-CaaD implicates two groups on the free enzyme with pK_a values of 7.6 and 9.3 for optimal activity. Likewise, two groups on CaaD with pK_a values of 7.6 and 9.2 are necessary for its optimal activity (*6*). It has been further determined for CaaD that βPro-1 is responsible for the pK_a value of 9.2 by ^{15}N NMR titration of the uniformly ^{15}N-labeled CaaD and its αP1A mutant (*6*). The ^{15}N NMR titration of the heterohexameric wild-type protein showed that the two amino-terminal prolines had pK_a values of 9.3 and 11.1. These pK_a values were assigned by an additional titration experiment using the αP1A mutant. In this experiment, a single pK_a value of 9.7 was observed, which can only correspond to the pK_a value of the βPro-1. While the pK_a value of Pro-1 in *cis*-CaaD has not been directly measured by NMR spectroscopy, it can be inferred from these results that the kinetic pK_a value of 9.3 is due to Pro-1, thereby implicating Pro-1 of *cis*-CaaD as a general acid catalyst at cellular pH.

These observations make *cis*-CaaD, along with CaaD, the only two known enzymes in the tautomerase superfamily where Pro-1 functions as a general acid catalyst.[4] In the other five characterized tautomerase superfamily members, Pro-1 functions as a general base catalyst and mediates a reversible enol−keto tautomerization reaction (*11*, *17*, *19*−*21*). This group includes two bacterial isomerases, 4-OT and 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI) (*19*), the mammalian cytokine, macrophage migration inhibitory factor (MIF), which exhibits a phenylpyruvate tautomerase

[3] An addition−elimination mechanism is a possible alternative mechanism. In this mechanism, nucleophilic attack by an active site residue at the C-3 position of *cis*-**1** would be followed by the elmination of the halide and generate an alkenyl-enzyme intermediate. Hydrolysis would release the enzyme and product. A single turnover experiment in $H_2{}^{18}O$ could be used to determine whether such a mechanism is operative. However, the outcome of this experiment would be ambiguous for the *cis*-CaaD system due to the rapid formation of the hydrate of **5**, which would ensure the presence of ^{18}O-labeled product (*2*, *3*, *18*). It should also be noted that *cis*-CaaD shares no sequence identity with other dehalogenases known to proceed through a covalent intermediate.

[4] A recent ^{15}N NMR titration of Pro-1 of malonate semialdehyde decarboxylase (MSAD), which converts **5** to acetaldehyde (*18*), shows that it has a pK_a of 9.2, making MSAD the third known tautomerase superfamily member with this pK_a value for the N-terminal proline (G. J. Poelarends, H. Serrano, W. H. Johnson, Jr., D. W. Hoffman, and C. P. Whitman, unpublished results).

activity (*20*), and two 4-OT homologues found in *Bacillus subtilis* (designated YwhB) (*11*) and *Escherichia coli* (designated YdcE) (*21*). Pro-1 can function as a general base in these enzyme-catalyzed reactions because it has an unusually low pK_a value. For 4-OT, the pK_a value is ~6.4, which is due primarily to the presence of Pro-1 in a hydrophobic environment although the influence of a nearby positively charged arginine (Arg-39) cannot be excluded (*14*, *17*, *22*). In contrast, the crystal structure of CaaD shows that Pro-1 resides in a hydrophilic environment, which may be at least partially responsible for a pK_a value that is more typically observed for an amino-terminal group (*5*).

The difference between the active site environments of Pro-1 in 4-OT and in the two dehalogenases likely reflects the different reactions carried out by these enzymes. In CaaD and *cis*-CaaD, water must be present in the active site so that a hydration reaction can be carried out (*1*, *2*, *5*). The 4-OT-catalyzed reaction as well as those carried out by CHMI, MIF, YwhB, and YdcE does not use water as a reactant (*11*, *17*, *19*−*21*). Presumably, the environment of Pro-1 in *cis*-CaaD is comparable to that of CaaD, which results in the equivalent pK_a values. In the presence of saturating amounts of substrate, the observed pK_a value for the general acid catalyst in *cis*-CaaD decreases to 8.6, in contrast to CaaD where it increases to 9.8 (*6*). If this pK_a corresponds to Pro-1, then substrate binding could perturb its local environment and cause a decrease in the pK_a value. It is also possible that saturation of either enzyme with substrate exposes another residue on the enzyme and that titration of this residue is responsible for the decrease in pK_a (*cis*-CaaD) or the increase in pK_a (CaaD).

The identity of the residue with the pK_a of 7.6 in *cis*-CaaD is not known. For CaaD, it has been speculated that the corresponding pK_a of 7.6 is due to αGlu-52 (*6*). The same may hold true for *cis*-CaaD, which would make Glu-114 responsible for the observed pK_a value. If these pK_a values do correspond to the general base catalysts, they are clearly higher than the expected pK_a value of a glutamate group (pK_a ~ 4−5). Under saturating conditions, the observed pK_a value for *cis*-CaaD decreases to 6.6 while that of CaaD may have decreased to a value below 6.0. In the free enzymes, the glutamates may be buried in a hydrophobic environment, which could partially account for their high pK_a values. If these same residues (i.e., αGlu-52 and Glu-114) are responsible for the observed pK_a values in the saturated enzymes, then the decrease in the pK_a values may reflect the fact that the glutamates are now exposed to the hydrophilic environment. The possibility that another residue is responsible for the observed kinetic pK_a cannot be ruled out. The assignments of these pK_a values are currently under investigation.

While the pH−rate profiles are consistent with the involvement of a general acid and a general base catalyst in the mechanism of *cis*-CaaD, there are likely more residues involved in the activation of the water molecule, as demonstrated by the results of previous mutagenesis experiments (*3*). It was determined that replacing αGlu-52 in CaaD with a glutamine generates an enzyme with no residual activity (*5*). In contrast, the E114Q mutant of *cis*-CaaD retains a significant amount of activity, showing only an 8-fold decrease in the k_{cat}/K_m value measured using *cis*-**1** (*3*). The presence of a second basic residue in *cis*-CaaD could result in the partial activation of water and account for the residual activity.

Mechanistic Analysis of the Inactivation of cis-CaaD. To identify additional catalytic residues and to delineate further similarities and differences between the two enzymes, the enantiomers of oxirane-2-carboxylate (**6**) were examined as potential inhibitors. Oxirane-containing compounds are well-known irreversible inhibitors of several enzymes and have been used to identify active site residues (*23*−*25*). Inactivation involves an acid and base catalyst and generally occurs by one of two mechanisms. In one mechanism, an acid catalyst protonates the oxygen atom, which results in ring opening and the formation of a carbocation intermediate (*23*). Subsequently, the base catalyst attacks the carbocation and becomes alkylated. In a second mechanism, ring opening is initiated by the nucleophilic attack of the base catalyst at one of the two carbon atoms of the oxirane ring (*24*, *25*). Ring opening is facilitated by the presence of an acid catalyst, which polarizes the carbon−oxygen bond (*24*, *25*). These two mechanisms can sometimes be distinguished by the regiochemistry of the ring-opened product although geometric constraints within the active site may preclude such a determination (*24*, *26*). In the first mechanism, alkylation will occur at the more substituted carbon (C-2) due to the greater stability of the carbocation intermediate. In the nucleophilic substitution reaction, alkylation will generally occur at the less sterically hindered carbon (C-3).

It was initially anticipated that for both enzymes Pro-1 would function as the acid catalyst to assist in ring opening by protonation of the oxygen atom. The base catalyst, αGlu-52 or Glu-114, or another active site nucleophile could then be alkylated by attack at C-2 or C-3 of **6**. It was further anticipated that the two dehalogenases might display selectivity for one of the enantiomers. The results show that only *cis*-CaaD is irreversibly inhibited and only by (*R*)-oxirane-2-carboxylate. Inactivation is active-site-directed as demonstrated by the observation of saturation kinetics (using the racemic mixture) and protection from inactivation by the presence of substrate (*15*). On the basis of these two findings, it can be reasonably inferred that the (*R*)-oxirane-2-carboxylate forms a dissociable complex at the active site, which leads to inactivation. The complex is analogous to the one formed between substrate and enzyme.

It has also been determined that the (*S*)-**6** binds to the active site of *cis*-CaaD but does not cause irreversible inhibition of the enzyme. Hence, the presence of the (*S*)-enantiomer in the racemic mixture probably affords some protection (particularly at the higher concentrations), which could be partially responsible for the slower rate of inactivation of *cis*-CaaD by the racemic mixture. The (*R*)- and (*S*)-enantiomers of **6** bind somewhat more tightly to the active site of CaaD, as assessed by the K_I values, but again do not inactivate the enzyme. It has also been demonstrated that the oxiranes are not hydrolyzed to their ring-opened products.

The sum of these observations indicates that the orientation of the (*R*)-enantiomer of **6** in the active site of *cis*-CaaD is a key factor in its ability to alkylate and thereby inactivate the enzyme. The two arginine residues (Arg-70 and Arg-73) clearly play a role in placing the oxirane in the proper orientation, as the absence of either one results in a mutant enzyme that cannot be alkylated. In one possible binding mode, the two arginine residues could interact with the C-1

carboxylate group of **6** in a fashion analogous to their presumed binding of the C-1 carboxylate group of substrate where each arginine interacts with a carboxylate oxygen. In an alternative binding mode, one arginine could interact with one or both oxygens of the C-1 carboxylate group and the second arginine could interact with the oxygen atom of the oxirane. The binding mode must position Pro-1 near C-2 or C-3 of **6** because Pro-1 is the residue covalently modified on the enzyme. Moreover, Glu-114, the presumed general base catalyst that is not required for the irreversible inhibition of *cis*-CaaD, is not proximal to C-2 or C-3 of **6** in this binding mode.

The irreversible inhibition of *cis*-CaaD by the covalent modification of Pro-1 was surprising because the enzyme is present primarily in a form where Pro-1 is a charged species not subject to alkylation. Assuming that Pro-1 has a pK_a of 9.3, only ~1.0% of *cis*-CaaD is in a state where Pro-1 is a conjugate base under the conditions of the inactivation experiments (pH 7.3). However, alkylation of Pro-1 would shift the equilibrium and place more of the enzyme in the reactive state. In this manner, all of the enzyme could be alkylated and thereby irreversibly inhibited.

The final questions concern the mechanism of alkylation and how the oxygen atom of the oxirane ring is protonated. As noted above, there are two possible mechanisms that could lead to the alkylation of Pro-1. One involves a carbocation intermediate, and the second entails a nucleophilic substitution reaction (*23*−*25*). The regiochemistry of the reaction is not yet known so a distinction between mechanisms cannot be made on this basis. While Pro-1 is the nucleophile in both mechanisms, there are several potential sources for a proton that would assist in ring opening. One source is a group on the enzyme such as one of the two arginines (Arg-70 or Arg-73) known to be required for the inactivation process or another unidentified residue. Other sources include water or buffer, which are also known to assist in the ring opening of epoxides (*27*, *28*).

A number of these questions can be answered by an analysis of the crystal structures of the inactivated *cis*-CaaD and that of a CaaD·(*R*)-**6** complex. In addition to providing valuable insights into the inactivation process, a comparison of these structures may suggest a structural basis for the reactivity of (*R*)-**6**. Moreover, this comparative analysis will shed further light on the structural basis for the similarities as well as the differences between the active sites of the two enzymes. These crystal structures are currently being pursued.

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