Inactivation of Cg10062, a *cis*-3-Chloroacrylic Acid Dehalogenase Homologue in *Corynebacterium glutamicum*, by (*R*)- and (*S*)-Oxirane-2-carboxylate: Analysis and Implications†

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ABSTRACT: (*R*)- and (*S*)-oxirane-2-carboxylate were determined to be active site-directed irreversible inhibitors of the *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD) homologue Cg10062 found in *Corynebacterium glutamicum*. Kinetic analysis indicates that the (*R*) enantiomer binds more tightly and is the more potent inhibitor, likely reflecting more favorable interactions with active site residues. Pro-1 is the sole site of covalent modification by the (*R*) and (*S*) enantiomers. Pro-1, Arg-70, Arg-73, and Glu-114, previously identified as catalytic residues in Cg10062, have also been implicated in the inactivation mechanism. Pro-1, Arg-70, and Arg-73 are essential residues for the process as indicated by the observation that the enzymes with the corresponding alanine mutations are not covalently modified by either enantiomer. The E114Q mutant slows covalent modification of Cg10062 but does not prevent it. The results are comparable to those found for the irreversible inactivation of *cis*-CaaD by (*R*)-oxirane-2-carboxylate with two important distinctions: the alkylation of *cis*-CaaD is stereospecific, and Glu-114 does not take part in the *cis*-CaaD inactivation mechanism. Cg10062 exhibits low-level *cis*-CaaD and *trans*-3-chloroacrylic acid dehalogenase (CaaD) activities, with the *cis*-CaaD activity predominating. Hence, the preference of Cg10062 for the *cis* isomer correlates with the observation that the (*R*) enantiomer is the more potent inactivator. Moreover, the factors responsible for the relaxed substrate specificity of Cg10062 may account for the stereoselective inactivation by the enantiomeric epoxides. Delineation of these factors would provide a more complete picture of the substrate specificity determinants for *cis*-CaaD. This study represents an important step toward this goal by setting the stage for a crystallographic analysis of inactivated Cg10062.

Cg10062 is a *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD)[1] homologue (~34% identical in sequence with *cis*-CaaD) found in *Corynebacterium glutamicum* (*1*). The physiological role and reaction of Cg10062 are unknown, and the gene has no clear genomic context. In contrast, *cis*-CaaD catalyzes the conversion of *cis*-3-chloroacrylic acid (**2**, Scheme 1) to malonate semialdehyde (**4**), which is one reaction in a catabolic pathway for the nematocide 1,3-dichloropropene (**1**) in coryneform bacterial strain FG41 (*2, 3*). An enzyme-catalyzed decarboxylation of **4** completes the overall transformation of **1** to acetaldehyde (**5**). *cis*-CaaD and its counterpart in *Pseudomonas pavonaceae* 170, *trans*-3-chloroacrylic acid dehalogenase (CaaD), which processes **3** to **4**, are highly specific for their substrates (*4, 5*).

Both Cg10062 and *cis*-CaaD are trimers composed of 149-amino acid monomers, and a sequence comparison shows that six key catalytic residues (Pro-1, His-28, Arg-70, Arg-73, Tyr-103, and Glu-114) in *cis*-CaaD are present in Cg10062 (*1, 6, 7*). In the proposed *cis*-CaaD mechanism, Glu-114 and Tyr-103 activate a water molecule for addition to C-3 of **2** (*1, 7*). His-28, Arg-70, and Arg-73 interact with the C-1 carboxylate group to bind and "activate" the substrate for the conjugate addition of water (*1, 7*). Pro-1 provides a proton to C-2, which completes the reaction and yields **4**.

Cg10062 and *cis*-CaaD are found in the same family in the tautomerase superfamily, the members of which are distinguished by a $\beta-\alpha-\beta$ building block and a catalytic Pro-1 (*1, 7*). Despite the presence of the key catalytic groups and the high degree of amino acid sequence similarity with *cis*-CaaD, Cg10062 is a poor *cis*-CaaD. It has a much lower catalytic efficiency, and it does not display absolute specificity for the *cis* isomer (*6*). These observations suggest that a fully functional *cis*-CaaD requires features beyond those already identified, and Cg10062 could be representative of the type of intermediate template that gave rise to *cis*-CaaD (*6*).

As part of an effort to determine a structural basis for the differences between the two enzymes, we examined (*R*)- and (*S*)-oxirane-2-carboxylate (**6**) as potential irreversible inhibitors of Cg10062. We have previously reported that *cis*-CaaD is irreversibly inactivated by (*R*)-**6**, due to the covalent modification of Pro-1 (*8*). The (*S*) enantiomer is not an irreversible inhibitor, and neither enantiomer inactivates

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[1] Abbreviations: *cis*-CaaD and CaaD, *cis*- and *trans*-3-chloroacrylic acid dehalogenase, respectively; ESI-MS, electrospray ionization mass spectrometry; MALDI-PSD, matrix-assisted laser desorption ionization postsource decay; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MSAD, malonate semialdehyde decarboxylase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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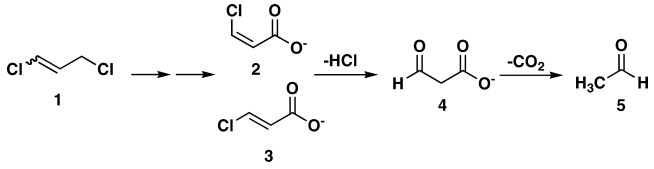
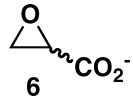
Scheme 1



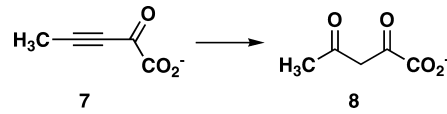
CaaD. In accord with the relaxed substrate specificity and preference for **2**, we find that Cg10062 is irreversibly inactivated by both enantiomers, with the (*R*) enantiomer being more potent. Inactivation is due to covalent modification of Pro-1. Pro-1, Arg-70, and Arg-73 are essential for the inactivation of Cg10062, whereas Glu-114 may play a role but not an essential one. These observations parallel those found for *cis*-CaaD, but the lack of stereospecificity and the participation of Glu-114 distinguish the inactivation mechanism for Cg10062 from that of *cis*-CaaD. Hence, the inactivation mechanisms could be similar but may result from different orientations in the active site. These results provide the necessary foundation for a crystallographic analysis of Cg10062 inactivated by (*R*)- and (*S*)-**6** so that these orientations can be determined.



MATERIALS AND METHODS

Materials. All reagents, buffers, and solvents were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), Spectrum Laboratory Products, Inc. (New Brunswick, NJ), or EM Science (Cincinnati, OH), unless noted otherwise. Literature procedures were used for the syntheses of (*R*)- and (*S*)-**6** (*9*) and 2-oxo-3-pentynoate (**7**) (*10*). *cis*-CaaD, malonate semialdehyde decarboxylase (MSAD), Cg10062, and the Cg10062 mutants (P1A, R70A, R73A, and E114Q) (*6*) were purified to homogeneity, as assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), according to published procedures (*1, 6, 1*). The construction and characterization of the Cg10062 mutants are reported elsewhere (*6*). Prepacked PD-10 Sephadex G-25 columns were purchased from Biosciences AB (Uppsala, Sweden). Endoproteinase Glu-C (protease V-8) was obtained from F. Hoffmann-La Roche, Ltd. (Basel, Switzerland).

General Methods. Protein was analyzed by SDS−PAGE under denaturing conditions on gels containing 15% polyacrylamide (*12*). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined by the method of Waddell (*13*). Kinetic data were obtained on a Hewlett-Packard 8452A diode array spectrophotometer or an Agilent 8453 UV−visible spectrophotometer. The kinetic data were fitted by nonlinear regression data analysis using Grafit (Erithacus Software Ltd., Horley, U.K.) obtained from Sigma Chemical Co. Cg10062 activity was determined by following the absorbance increase at 296 nm ($\varepsilon = 7000\ M^{-1}\ cm^{-1}$), which corresponds to formation of acetopyruvate (**8**) by the Cg10062-catalyzed hydration of **7** (*1, 5, 6*). Typically, Cg10062 preparations used in these studies have a K_m of 4.2 ± 0.3 mM and a k_{cat} of 0.16 ± 0.01 s^{-1} using **7**. *cis*-CaaD activity was measured by following the absorbance decrease at 224 nm ($\varepsilon = 2900\ M^{-1}\ cm^{-1}$), as described elsewhere (*1*). Mass spectral analyses were carried out using the indicated instruments, which are housed in the Analytical Instrumentation Facility Core in the College of Pharmacy at The University of Texas.



*Irreversible Inhibition of Cg10062 by (R)- and (S)-***6.** The time-dependent inactivation of Cg10062 by (*R*)- and (*S*)-**6** was characterized using a previously described protocol (*8*), with the following modifications. Varying concentrations of (*R*)-**6** (0.99−33.0 mM) or (*S*)-**6** (4.8−56.5 mM) were incubated with the enzyme in 20 mM Na_2HPO_4 buffer (pH 9.0) at 22 °C. The initial incubation mixtures (total volume of 110 μL) were made up in 1.5 mL Eppendorf micro test tubes and contained 100 μM Cg10062 (based on a monomer concentration). Aliquots (10 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 using **7**. Assays were initiated by the addition of **7** (4 μL from a 365 mM stock solution) to give a final concentration of 1.5 mM for **7** in all experiments. Stock solutions of **7** were made up in 100 mM Na_2HPO_4 buffer (pH ∼9), and the pH was slowly adjusted to 7.3 via the addition of 2 μL aliquots of a 1 M NaOH solution. Stock solutions (100 mM) of (*R*)- or (*S*)-**6** were made up in 100 mM NaH_2PO_4 buffer (pH 7.3). For experiments using the (*R*) enantiomer, rates were monitored for 120 s, and for experiments using the (*S*) enantiomer, rates were monitored for 200 s. The initial time point ($t = 0$) corresponded to the rate measured for an aliquot removed before the addition of (*R*)- or (*S*)-**6**. This activity was defined as 100% activity. The addition of inhibitor [1−50 μL for (*R*)-**6** and 5−130 μL for (*S*)-**6**] diluted the enzyme so that the final enzyme concentration ranged from 67 to 99 μM for the (*R*) enantiomer and from 44 to 95 μM for the (*S*) enantiomer. Hence, for each aliquot removed, the observed rate was divided by the enzyme concentration (in the assay mixture) and the resulting rate was divided by that obtained for 100% activity. The k_{obs} values were plotted against the initial inhibitor concentrations, and the kinetic parameters (K_I and k_{inact}) were determined as described elsewhere (*14, 15*).

*Protection of Cg10062 from Inactivation by (R)- and (S)-***6.** The incubation mixtures for the substrate protection studies were made up as described above and elsewhere (*8*), with the following modifications. Accordingly, Cg10062 (100 μM based on a monomer concentration) was incubated with varying concentrations of **7** (0−5.25 mM) in 20 mM NaH_2PO_4 buffer (pH 7.3) at 22 °C. After a 30 s interval, a fixed concentration of (*R*)- or (*S*)-**6** (0.4 or 4.0 mM, respectively) was added to the mixture. Aliquots (10 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.

*Irreversibility of Inactivation of Cg10062 by (R)- and (S)-***6.** Three samples were made up containing ∼1.5 mg of enzyme (80 μL of a 18.5 mg/mL solution) and a sufficient quantity of 20 mM Na_2HPO_4 buffer (pH 9.0) to give a final volume of 990 μL. Two samples were treated with (*R*)- or

(*S*)-**6** [10 μL from a 100 mM stock solution in 100 mM NaH_2PO_4 buffer (pH 7.3)], and a third one was treated with 100 mM NaH_2PO_4 buffer (pH 7.3, 10 μL). After a 5 day incubation period at 4 °C, an aliquot (200 μL) was removed from the control and the (*R*)-**6**-modified Cg10062 sample and subjected to Sephadex G-25 chromatography as described previously (*5*). Subsequently, a solution was made up from each set of fractions such that a final concentration of 2 μM enzyme (~520 μL of a 0.26 mg/mL solution) was obtained in 3.5 mL of 20 mM Na_2HPO_4 buffer (pH 9.0). A 1 mL aliquot was removed from each solution and assayed for activity using **7**. Activity assays were initiated by the addition of **7** (4 μL from a 365 mM stock solution made up as described above) to give **7** at a final concentration of 1.5 mM. The remaining solution (~2.5 mL) was stored at 4 °C and assayed after an additional 3 day incubation period. The sample containing Cg10062 and (*S*)-**6** was incubated for 10 days at 4 °C and then processed in a comparable manner.

*Irreversible Inhibition of cis-CaaD by (R)-***6.** The time-dependent inactivation of *cis*-CaaD by (*R*)-**6** was assessed using varying concentrations of inhibitor (0−14 mM) and enzyme (20 μM based on a monomer concentration) in 20 mM Na_2HPO_4 buffer (pH 9.0) at 22 °C, as described elsewhere (*8*). The incubation mixtures (total volume of 101−114 μL) were made up in 1.5 mL Eppendorf micro test tubes. Aliquots (10 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 using **2** at a final concentration of 200 μM. The initial time point ($t = 0$) corresponded to the aliquot removed immediately after the addition of (*R*)-**6** to the incubation mixture. The *cis*-CaaD activity measured for this aliquot was defined as 100% activity. Activity assays were initiated by the addition of an aliquot of **2** (4 μL) removed from a 50 mM stock solution made up in 100 mM Na_2HPO_4 buffer (pH 9.1). The pH of the stock solution was adjusted to ~7.5 via the addition of small quantities of 1 M NaOH.

*Mass Spectral Analysis of Cg10062 and Mutants Incubated with (R)- and (S)-***6.** The covalently modified Cg10062 samples were prepared for mass spectral analysis by the incubation of the enzyme with (*R*)- or (*S*)-**6** in 20 mM Na_2HPO_4 buffer (pH 9.0) as follows. Each sample contained ~1.5 mg of enzyme (~81 μL of a 18.5 mg/mL solution) and a sufficient quantity of 20 mM Na_2HPO_4 buffer (pH 9.0) to give a final volume of 990 μL. The samples were treated with (*R*)- or (*S*)-**6** [10 μL from a 100 mM stock solution of (*R*)- or (*S*)-**6** in 100 mM NaH_2PO_4 buffer (pH 7.3)]. A control sample was made up similarly, but the enzyme was treated with a 10 μL portion of buffer. Subsequently, the mixture containing Cg10062 and (*R*)-**6** was incubated at 4 °C for 24 h and analyzed. The control sample and the sample containing Cg10062 and (*S*)-**6** were incubated at 4 °C for 10 days and aliquots removed and analyzed after 24 h, 48 h, 5 days, and 10 days. The P1A, R70A, R73A, and E114Q mutants of Cg10062 were incubated separately with (*R*)- or (*S*)-**6** in 20 mM Na_2HPO_4 buffer (pH 9.0) as follows. Samples contained ~1.75 mg of enzyme (~100 μL of a 17.5 mg/mL solution) and a sufficient quantity of the 20 mM Na_2HPO_4 buffer to give a final volume of 495 μL. The samples were treated with (*R*)- or (*S*)-**6** [5 μL from a 100 mM stock solution of (*R*)- or (*S*)-**6** in 100 mM NaH_2PO_4 buffer (pH 7.3)]. The mixtures were incubated at 4 °C for 10 days and aliquots removed and analyzed after 24 h, 5 days, and 10 days. Samples for electrospray ionization mass spectrometry (ESI-MS) analysis were made up as described previously (*5*) and analyzed using an LCQ electrospray ion trap mass spectrometer (Thermo, San Jose, CA).

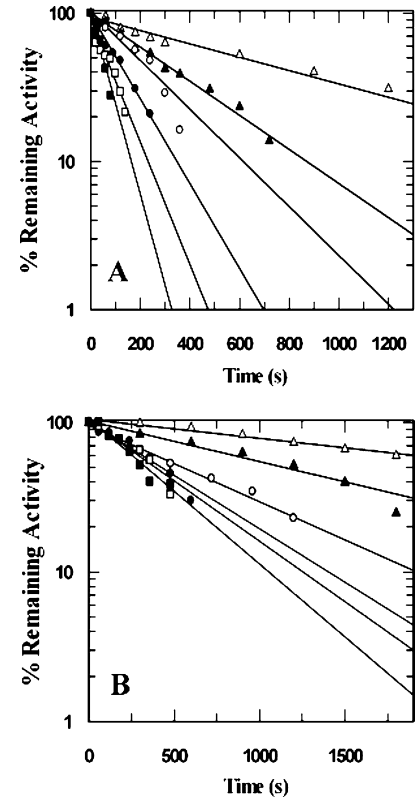


FIGURE 1: Time-dependent inactivation of Cg10062 by (*R*)- and (*S*)-**6**. (A) A logarithmic plot of the percent Cg10062 activity remaining as a function of time using varying amounts of (*R*)-**6** [(△) 0.99, (▲) 2.9, (○) 4.8, (●) 6.5, (□) 9.1, and (■) 33.0 mM]. (B) A logarithmic plot of the percent Cg10062 activity remaining as a function of time using varying amounts of (*S*)-**6** [(△) 4.8, (▲) 9.1, (○) 16.7, (●) 23.1, (□) 37.5, and (■) 56.5 mM]. For purposes of clarity, the rates obtained for six experiments are shown. The data from all of the experiments were used to calculate k_{obsd}.

*Peptide Mapping of Cg10062 Inactivated by (R)- and (S)-***6.** Three samples were made up containing ~1 mg of enzyme (39 μL of a 26.5 mg/mL solution) and a sufficient quantity of 20 mM NaH_2PO_4 buffer (pH 7.3) to give a final volume of 500 μL. Two samples were treated with (*R*)- or (*S*)-**6** [5 μL from a 100 mM stock solution in 100 mM NaH_2PO_4 buffer (pH 7.3)], and a third sample was treated with buffer (5 μL). After a 24 h incubation period at 4 °C, the samples were subjected to Sephadex G-25 chromatography as described previously (*5*), yielding three sets of fractions containing modified Cg10062 [by (*R*)- or (*S*)-**6**] or unmodified Cg10062. A sufficient quantity of protein was removed from the fraction containing the highest concentration of protein [now in 100 mM NH_4HCO_3 buffer (pH 8.0)] to give ~27 μg of enzyme, which was diluted into the necessary quantity of 100 mM NH_4HCO_3 buffer to yield a final volume of 45 μL. After the addition of a 5 μL aliquot of 10 M guanidine HCl, the three samples were incubated for 1 h at 37 °C. The protein samples were then incubated for an additional 48 h at 37 °C with sequencing grade protease V-8 (2 μL of a 10 mg/mL stock solution made up in water) (*16*). Subsequently, the 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 (*5*). Selected ions in the samples were also subjected to MALDI postsource decay (PSD) analysis using the protocol described elsewhere (*5, 17*).

*Mass Spectral Analysis of cis-CaaD Incubated with (S)-***6** *and MSAD Incubated with (R)- and (S)-***6.** A sample of *cis*-CaaD was made up as described above for Cg10062 and treated with (*S*)-**6** [10 μL from a 100 mM stock solution in 100 mM NaH_2PO_4 buffer (pH 7.3)]. Similarly, samples of MSAD were made up and treated with (*R*)- and (*S*)-**6** [10 μL from 100 mM stock solutions in 100 mM NaH_2PO_4 buffer (pH 7.3)]. The samples were incubated at 4 °C for 10 days and aliquots removed and analyzed after 24 h, 5 days, and 10 days as noted in the text. The samples were prepared for mass spectral analysis as described previously (*5*) and subjected to ESI-MS analysis.

RESULTS

*Time-Dependent Inactivation of Cg10062 by (R)- and (S)-***6.** Cg10062 is irreversibly inactivated by both (*R*)- and (*S*)-**6** in a time-dependent first-order process (Figure 1A,B). The k_{obs} values from 18 experiments using (*R*)-**6** and the k_{obs} values from 15 experiments using (*S*)-**6** were plotted versus the initial inhibitor concentrations and fit to a rectangular hyperbola.[2] The values of K_I and k_{inact} obtained from the plot for (*R*)-**6** (Figure 2A) were 14.0 ± 2.1 mM and 0.021 ± 0.001 s^{-1}, respectively. The values obtained from the plot for (*S*)-**6** (Figure 2B) were 71.2 ± 12.7 mM and 0.005 ± 0.001 s^{-1}, respectively. Assuming K_I is an estimate of binding affinity, these values indicate that (*R*)-**6** binds more tightly (~5.1-fold) at the active site than the (*S*) enantiomer and that at saturating concentrations of inhibitor, the (*R*) enantiomer is the more potent inactivator (~4.2-fold).

To compare directly the inactivation of Cg10062 with that of *cis*-CaaD, the kinetic parameters for the inactivation of *cis*-CaaD by (*R*)-**6** were determined again. Accordingly, the k_{obs} values from 18 experiments using *cis*-CaaD and (*R*)-**6** were fit to a rectangular hyperbola. The values of K_I and k_{inact} obtained from the plot for (*R*)-**6** (Figure 2C) were 10.8 ± 1.6 mM and 0.20 ± 0.02 s^{-1}, respectively.[3] Although (*R*)-**6** binds comparably to both enzymes (as indicated by the similar K_I values), the (*R*) enantiomer is an approximately 9.5-fold more potent inhibitor of *cis*-CaaD than it is of Cg10062. As noted elsewhere, the (*S*) enantiomer does not inactivate *cis*-CaaD but functions as a weak competitive inhibitor (*8*).

Both plots for the inactivation of Cg10062 show saturation kinetics, indicating that inactivation occurs by the prior formation of a dissociable complex between enzyme and inhibitor at the active site (*18*). Further evidence for binding at the active site comes from substrate protection studies using **7**, which at four different concentrations slows the inactivation of Cg10062 by (*R*)-**6**, and at two different concentrations slows the inactivation of Cg10062 by (*S*)-**6** (Figure 3A,B) (*18*).

The rates for the inactivation of Cg10062 by (*R*)- and (*S*)-**6** in the substrate protection studies (e.g., Figure 3 in the absence of **7**) differ from the corresponding ones in Figure 1. For example, 4 mM (*S*)-**6** results in ~20% remaining activity after 1500 s (Figure 3B), but 4.8 mM (*S*)-**6** results in ~60% remaining activity after 1500 s (Figure 1B). This discrepancy is likely due to the fact that the remaining activity was determined using different preparations of **7**. The variable quality and decomposition of **7** preclude an accurate determination of its concentration in solution. The conclusions do not change.

The irreversible nature of inactivation was demonstrated by the observation that Cg10062 inactivated by either (*R*)- or (*S*)-**6** did not regain activity after gel filtration on a PD-10 Sephadex G-25 column. After 5 days, Cg10062 incubated with (*R*)-**6** had no residual activity (using **7**) compared to a control. Gel filtration and an additional 3 day incubation period at 4 °C did not result in recovery of activity. Incubation of Cg10062 with (*S*)-**6** completely inactivated the enzyme after a 10 day interval. Once again, gel filtration and an additional 3 day incubation period at 4 °C did not result in the recovery of activity.

*Mass Spectral Analysis of Cg10062 Treated with (R)- and (S)-***6.** Both the (*R*)- and (*S*)-**6**-inactivated Cg10062 were subjected to ESI-MS analysis and the spectra compared to that of the wild type. An 11:1 ratio of inhibitor to enzyme concentration (based on a monomer molecular mass) was used. For Cg10062 inactivated by the (*R*) enantiomer, the spectrum acquired after a 24 h incubation period exhibits a major signal corresponding to a mass of 17190 ± 2 Da (data not shown). A similar spectrum is obtained after a 48 h incubation period. The signal corresponds to the mass of Cg10062 (17092 ± 2 Da) modified by the covalent attachment of a species with a mass of 88 Da. There is no residual signal for unmodified Cg10062 indicating ~100% modification.

For Cg10062 inactivated by the (*S*) enantiomer, the spectrum acquired after a 24 h incubation period exhibits signals corresponding to masses of 17092 ± 2 and 17190 ± 2 Da (data not shown). The signal at 17092 Da corresponds to that of Cg10062, and the signal at 17190 Da corresponds to Cg10062 modified by the covalent attachment of the species with a mass of 88 Da. The intensities of the signals suggest 15−30% modification (in two separate runs). The same spectrum is obtained after a 48 h incubation period, where the intensities of the signals now suggest ~45% modification. After 5 days, the intensities of the signals suggest 60−70% modification. After 10 days, the signal at 17190 Da predominates. The relative intensities of the signals suggest ~90% modification.

For both (*R*)- and (*S*)-**6**-inactivated Cg10062, the increase in mass corresponds to the expected molecular mass of **6** as its ring-opened derivative (*7, 8*). The mass does not distinguish between a 2- or 3-hydroxypropanoate species

[2] In some cases, a better fit of the data can be obtained using the Hill equation, as found in Grafit. The K_I and k_{inact} values obtained for the inactivation of Cg10062 by (*R*)- and (*S*)-**6** and *cis*-CaaD by (*R*)-**6** by fitting the data to the Hill equation and the resulting plots are provided as Supporting Information. The resulting fits could suggest positive cooperativity and may indicate that the inactivation of one subunit accelerates the rate of inactivation for the two remaining subunits. Positive (or negative) cooperativity has not been detected for Cg10062 or *cis*-CaaD by steady state kinetics.

[3] In a previous report (*8*), the concentrations of **6** were calculated on the basis of a molecular mass of 88 Da, which corresponds to the free acid. Hence, the actual concentrations are lower than the reported concentrations (*8*). The concentrations in this report take into account the fact that **6** is a potassium salt.

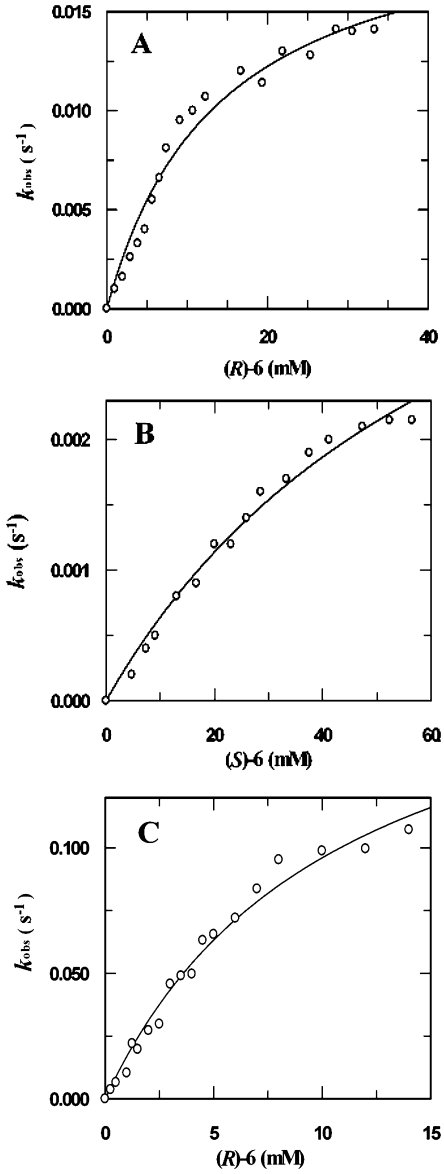


FIGURE 2: Plot of k_{obsd} values obtained for the inactivation of Cg10062 by (*R*)- and (*S*)-**6** and *cis*-CaaD by (*R*)-**6** vs the concentration of inactivator. (A) k_{obsd} values for 18 experiments using (*R*)-**6** and Cg10062 vs the concentration of (*R*)-**6**. (B) k_{obsd} values for 15 experiments using (*S*)-**6** and Cg10062 vs the concentration of (*S*)-**6**. (C) k_{obsd} values for 18 experiments using (*R*)-**6** and *cis*-CaaD vs the concentration of (*R*)-**6**. The data from all of the experiments were used to calculate k_{inact} and K_I, which are reported in the text.

attached to the enzyme. However, for both enantiomers, Cg10062 shows only a single site of modification.

*Mass Spectral Analysis of cis-CaaD Treated with (S)-***6** *and MSAD Treated with (R)- and (S)-***6.** In a previous report, covalent modification of *cis*-CaaD by (*S*)-**6** was not detected for a sample analyzed after a 24 h incubation period (*8*). In view of the lengthy incubation times required for near-complete covalent modification of Cg10062 by the (*S*) enantiomer, *cis*-CaaD was incubated with (*S*)-**6** for 10 days at a ratio of 11:1 (inhibitor:enzyme). The spectrum showed only a signal corresponding to the mass of unmodified *cis*-CaaD, confirming that *cis*-CaaD is not covalently modified by (*S*)-**6**. The inactivation of MSAD by (*R*)- and (*S*)-**6** was not previously reported. At ratios of 7:1 and 11:1 (inhibitor:enzyme), the spectra for MSAD samples incubated with (*R*)-

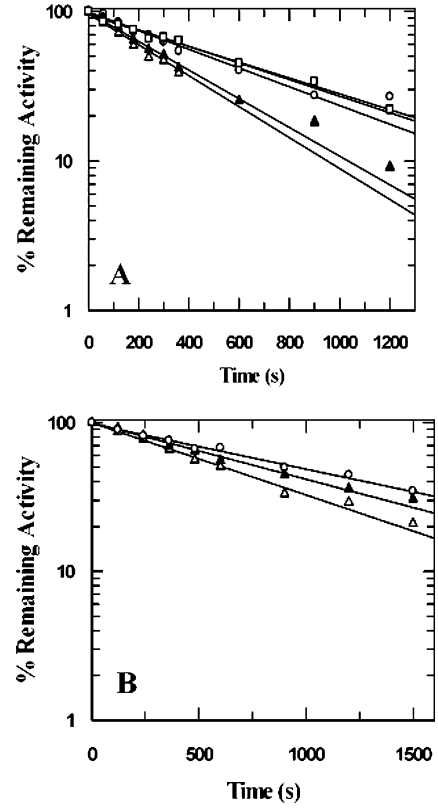


FIGURE 3: Protection of Cg10062 from inactivation by (*R*)- and (*S*)-**6** using substrate **7**. (A) Cg10062 (100 μM) was incubated with four different concentrations of **7** [(△) 0, (▲) 1.0, (○) 2.1, (●) 3.5, and (□) 5.2 mM] for 30 s before the addition of (*R*)-**6** (0.4 mM). (B) The same quantity of Cg10062 was incubated with two different concentrations of **7** [(△) 0, (▲) 1.7, and (○) 3.5 mM] for 30 s before the addition of (*S*)-**6** (4.0 mM).

or (*S*)-**6** for a 10 day period show only signals at 14107 ± 2 Da (data not shown). This mass corresponds to the expected mass of the MSAD monomer (*11*) and indicates that MSAD is not covalently modified by either enantiomer of **6**.

*Mass Spectral Analysis of Cg10062 Mutants Treated with (R)- and (S)-***6.** Four active site mutants of Cg10062 (P1A, R70A, R73A, and E114Q) were incubated in individual reaction mixtures with (*R*)- and (*S*)-**6** for 10 days at 4 °C (*6*). Mass spectral analysis of the reaction mixtures containing the P1A, R70A, and R73A mutants showed that each one produced signals (17066, 17007, and 17006 ± 2 Da, respectively) corresponding to the expected molecular masses of the unmodified mutants (*6*). Thus, Pro-1, Arg-70, and Arg-73 are essential for covalent modification of Cg10062 by either (*R*)- or (*S*)-**6**. Mass spectral analysis of the reaction mixture containing the E114Q mutant revealed a more complex situation. Analysis of the mixture containing (*R*)-**6** and the E114Q mutant (7:1 ratio) after 24 h exhibited two signals corresponding to the masses of the unmodified enzyme (17092 ± 2 Da) and the modified enzyme (17180 ± 2 Da). The relative intensities suggested 40% covalent modification. Using the same ratio of inhibitor to enzyme, wild-type Cg10062 was completely modified by (*R*)-**6** after 24 h. After 5 days, mass spectral analysis showed complete covalent modification of the E114Q mutant by (*R*)-**6**. Analysis of the mixture containing (*S*)-**6** and the E114Q mutant (7:1 ratio) at 24 h showed only a mass corresponding to unmodified enzyme. After 5 days, a second signal appeared corresponding to the mass of the modified enzyme.

Table 1: Peptides Identified in the Protease V-8 Digestion Mixture of Unmodified and (*R*)- and (*S*)-**6**-Modified Cg10062

peptide fragment	calculated mass (Da)[a]	observed mass[b] of Cg10062 (Da)	observed mass[b] of (*R*)- and (*S*)-**6**-modified Cg10062 (Da)	
^{1}P−^{15}E	1896.15	1895.69	1983.86	1983.74
^{31}L−^{41}E	1742.05	1741.75	1741.89	1741.80
^{61}N−^{75}E	1767.96	1767.67	1767.84	1767.76
^{81}L−^{88}E	985.19	985.48	985.55	985.52
^{115}Y−^{126}E	1422.57	1422.47	1422.58	1422.53

[a] The monoisotopic singly charged masses are predicted from analysis of the translated amino acid sequence of the *Cg10062* gene (corresponding to Cg10062). [b] The observed masses correspond to MH^+.

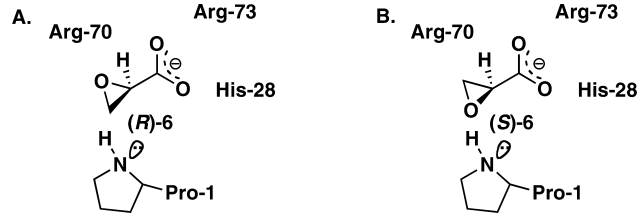
The relative intensities of the signals indicated 20% modification. After 10 days, mass spectral analysis suggested 45% covalent modification of the E114Q mutant by the (*S*) enantiomer. Using the same ratio of inhibitor to enzyme, wild-type Cg10062 was 60% modified by (*S*)-**6** after 10 days. Thus, in contrast to *cis*-CaaD, where Glu-114 is not required for the inactivation reaction, it plays a role in the alkylation of Cg10062 by (*R*)- and (*S*)-**6** because the E114Q mutant slows the inactivation reaction.

*Identification of Pro-1 as the Site of Modification by (R)- and (S)-***6.** ESI-MS analysis of incubation mixtures containing Cg10062 and (*R*)- or (*S*)-**6** showed covalent attachment of a species with a mass of 88 Da to the enzyme. The mass is consistent with modification of the enzyme by a 2- or 3-hydroxypropanoate species (*7, 8*). The modified residue was identified by incubating the three samples [unmodified Cg10062, (*R*)-**6**-modified Cg10062, and (*S*)-**6**-modified Cg10062] with endoproteinase Glu-C (protease V-8) and analyzing the resulting peptide mixtures by MALDI-MS. Although protease V-8 cleaves peptide bonds at the carboxylate side of glutamate and aspartate residues (leaving Glu or Asp as the C-terminal residue), glutamates are preferred (in 100 mM ammonium bicarbonate buffer at pH 8.0) (*16*).

Mass spectral analysis identified five major peptide species in all three samples corresponding to peptide bond cleavage at Glu-15, Glu-30, Glu-41, Glu-60, Glu-75, Glu-80, Glu-88, Glu-114, and Glu-126 (Table 1). Additional peptide species were identified in the (*R*)- and (*S*)-**6**-modified Cg10062 samples having masses of 1983.86 and 1983.74 Da, respectively. The mass corresponds to the Pro-1−Glu-15 peptide fragment covalently modified by **6**. The other four peptide fragments (Table 1) had not been modified.

The unmodified (1895.69 Da) and (*R*)- and (*S*)-**6**-modified fragments (1983.86 and 1983.74 Da, respectively) were subjected to MALDI-PSD analysis in an effort to locate the residue with the covalently attached species (*17*). The PSD spectrum of the unmodified peptide exhibited N-terminal sequence-specific fragment ions b_2 (199) and b_3 (362). The PSD spectrum of the modified peptide exhibited modified fragment ions b_2 (287), corresponding to a modified Pro-1−Thr-2 fragment, and b_4 (551), corresponding to the modified Pro-1−Thr-4 fragment. These observations narrowed the site of covalent attachment to Pro-1 or Thr-2. Although the PSD spectra of the unmodified and two modified fragments did not show a b_1 ion, corresponding to Pro-1 or modified Pro-1, a proline immonium ion (70) and a modified proline immonium ion (158) were present in the spectra for the (*R*)- and (*S*)-**6**-modified fragments. Hence, Pro-1 is the site of covalent modification by (*R*)- and (*S*)-**6**.

Scheme 2



DISCUSSION

cis-CaaD and CaaD are isomer-specific dehalogenases and represent two of the five known families in the tautomerase superfamily (*1*). Tautomerase superfamily members are characterized by their signature $\beta-\alpha-\beta$ building block and catalytic Pro-1 (*19–21*). The catalytic mechanisms of *cis*-CaaD and CaaD are largely the same (*1, 5, 7*). Both have a glutamate residue (Glu-114 and αGlu-52, respectively)[4] that activates water for attack at C-3 of the substrate (**2** and **3**, respectively), a pair of arginines (Arg-70 and Arg-73 and αArg-8 and αArg11, respectively) that interacts with the C-1 carboxylate group to bind and polarize the substrate, and an amino-terminal proline (Pro-1 and βPro-1, respectively) that adds a proton to the C-2 position to complete the conjugate addition of water. Two additional groups, His-28 and Tyr-103, are found in *cis*-CaaD. His-28 assists in substrate binding, and Tyr-103 assists in water activation. Cg10062 is a *cis*-CaaD family member that functions as a hydratase but a poor *cis*-CaaD. Despite the presence of the six residues that make up the core catalytic machinery, Cg10062 has a much lower catalytic efficiency than *cis*-CaaD and lacks absolute isomer specificity (*6*). A determination of the basis for the poor *cis*-CaaD activity of Cg10062 could provide insights into the elements required for a fully functional and highly specific *cis*-CaaD and suggest how *cis*-CaaD evolved.

(*R*)-Oxriane-2-carboxylate (**6**) was previously characterized as a stereospecific affinity label of *cis*-CaaD (*8*). Mass spectral and crystallographic analysis established that inactivation resulted from covalent modification of the Pro-1 nitrogen by (*R*)-2-hydroxypropanoate at the C-3 position (*7, 8*). Pro-1, Arg-70, and Arg-73 were identified as essential active site residues for the inactivation mechanism (*8*). The crystallographic analysis of the inactivated *cis*-CaaD implicated His-28 in the mechanism and suggested roles for Arg-70 and Arg-73 (*7*). On the basis of these observations, it was proposed that Arg-73 and His-28 interact with the carboxylate group and position (*R*)-**6** for covalent modification of Pro-1 (Scheme 2A). The side chain of Arg-70 or an Arg-70-bound water molecule functions as the proton donor for the epoxide ring opening reaction. This proposed orientation places the (*R*) enantiomer in position for the alkylation reaction. If the (*S*) enantiomer binds similarly, the epoxide ring is flipped so that the Pro-1 nitrogen cannot be alkylated (Scheme 2B).

[4] In contrast to *cis*-CaaD and Cg10062, which are trimers, CaaD is a heterohexamer consisting of three α-subunits and three β-subunits (*1, 4*). A functional CaaD active site is a heterodimer made up of an α-subunit and a β-subunit.

The presence of His-28 in *cis*-CaaD may be a determinant of the enzyme's vulnerability to alkylation (*7*). CaaD, which is not inactivated by either enantiomer, lacks this residue or an equivalent. As in the proposed binding mode for **3**, the carboxylate group of **6** may interact with the two arginines (αArg-8 and αArg-11) in the active site of CaaD, thereby preventing one of the arginines from serving as a necessary proton source to facilitate ring opening. We have also confirmed that MSAD is not inactivated by either enantiomer of **6**. Like CaaD, MSAD has two arginines residues (Arg-73 and Arg-75) but lacks His-28 or an equivalent. The two arginine residues of MSAD are proposed to interact with the substrate and may likewise interact with the carboxylate group of **6** and prevent one from functioning as a proton source. Evidently, there is not an alternate nearby proton source.

Oxirane-containing compounds have been used extensively as affinity labels of enzymes, and the subsequent characterization of the inactivated enzyme has provided much mechanistic insight (*22–24*). Ring opening, with the concomitant alkylation and inactivation, generally involves acid−base catalysis and proceeds by one of two mechanisms (*25*). In one mechanism, the acid catalyst protonates the oxygen of the oxirane ring (*25*). Ring opening produces a carbocation, which captures a nearby base catalyst at the more highly substituted carbon (*25*). In a second mechanism, the base catalyst attacks the less sterically hindered carbon of the oxirane in conjugation with polarization of the carbon−oxygen ring by the acid catalyst. The regiochemistry of the ring-opened product generated in the *cis*-CaaD reaction with (*R*)-**6** would suggest that the second mechanism is operative. However, it is unknown whether active site constraints played a role in the observed outcome (*23*).

The two key findings in this study are that both (*R*)- and (*S*)-**6** inactivate Cg10062 and that Glu-114 is an additional participant in the inactivation mechanism. Otherwise, the inactivation process parallels that observed for *cis*-CaaD, and for the (*R*) enantiomer, a similar mechanism can be envisioned. Accordingly, the carboxylate group would interact with His-28 and Arg-73, and the side chain of Arg-70 or an Arg-70-bound water molecule would interact with the oxirane oxygen to facilitate ring opening. Pro-1 is positioned to attack at C-3.[5] The lower potency [compared to the inactivation of *cis*-CaaD by (*R*)-**6**] suggests weaker binding and less favorable interactions with the key active site groups and may reflect some "wobble" in the active site with regard to inhibitor positioning and binding. Glu-114 might further position the epoxide for alkylation or maintain the position of one of the essential residues such that replacement with a glutamine slows the inactivation process.

There are at least two possible mechanistic explanations for the inactivation of Cg10062 by the (*S*) enantiomer. If it is assumed that the carboxylate group of (*S*)-**6** interacts with His-28 and Arg-73, one potentially interesting scenario involves a change in the regiochemistry of ring opening such that C-2 is the site of attachment. This scenario could involve another unknown residue functioning as the proton source for the oxirane oxygen. In a second scenario, the epoxide could bind in the active site of Cg10062 in a different orientation where the carboxylate group interacts with Arg-70 and Arg-73. Ring opening could occur at C-2 or C-3, and His-28 could function as a proton source. In both scenarios, the side chain of Glu-114 could favor a productive binding mode (i.e., one that results in alkylation) over a nonproductive one.

There are likely to be subtle differences between the active sites of *cis*-CaaD and Cg10062, and as a consequence, slight differences are observed between the inactivation mechanisms. The two enzymes are 34% identical in sequence (and 53% similar). The residues involved in binding and catalysis (Pro-1, His-28, Arg-70, Arg-73, Tyr-103, and Glu-114) as well as the other residues defining the *cis*-CaaD active site cavity (Thr-32, Thr-34, His-69, and Met-112) are mostly found in Cg10062. The two major exceptions are His-69 and the residues of a loop that connects the α-helix to the second β-strand in the $\beta-\alpha-\beta$ building block. In *cis*-CaaD, His-28 and His-69 interact with the hydroxyl group of Tyr-3. In Cg10062, His-69 is replaced with an isoleucine, which could affect this interaction and perhaps alter the position of His-28 and/or otherwise modulate the active site properties. These changes could contribute to the wobble in the positioning and binding of the inhibitor.

In addition, the loop residues in Cg10062 are significantly different from those found in *cis*-CaaD. In *cis*-CaaD, Leu-31 and Ala-39 anchor the loop, which consists of Thr-32, Gly-33, Thr-34, Gln-35, His-36, Phe-37, and Leu-38. In Cg10062, Leu-31 and Val-39 anchor the loop, which consists of Ala-32, His-33, Ala-34, Pro-35, Lys-36, Tyr-37, and Leu-38. Examination of the crystal structure of *cis*-CaaD inactivated by (*R*)-**6** shows interactions between the amide hydrogens of Phe-37 and Leu-38 and a carboxylate oxygen of the ring-opened product (2- or 3-hydroxypropanoate). These interactions may be precluded in Cg10062 due to the different N-terminal loop residues (i.e., residues 32−35). The absence of these interactions coupled with the potentially different properties of the loop may result in a less discriminating active site and allow binding of both enantiomers. Crystal structures of Cg10062 inactivated by the enantiomers of **6** will shed light on the basis for the differences in the inactivation mechanisms and are currently being pursued.

[5] In the proposed mechanism for Cg10062, Pro-1 functions as a general acid catalyst (*6*), raising the question of how the charged prolyl nitrogen is alkylated. The sequence similarity between the *cis*-CaaD and Cg10062 active sites suggests that the pK_a values for the prolyl nitrogens will be comparable (∼9.3) (*1, 8*). At the pH of the inactivation experiments (9.0), ∼33% of the enzyme is in a protonation state where the prolyl nitrogen can function as a nucleophile. Alkylation of the nitrogen perturbs the equilibrium and places an additional amount of enzyme into the nucleophilic and reactive form. In this manner, the enzyme can become entirely alkylated.

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SUPPORTING INFORMATION AVAILABLE

K_I and k_{inact} values obtained for the inactivation of Cg10062 by (*R*)- and (*S*)-**6** and *cis*-CaaD by (*R*)-**6** by fitting the data

to the Hill equation. This material is available free of charge via the Internet at http://pubs.acs.org.

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