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A Mutational Analysis of Active Site Residues in *trans*-3-Chloroacrylic Acid Dehalogenase

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Abstract

trans-3-Chloroacrylic acid dehalogenase (CaaD) catalyzes the hydrolytic dehalogenation of *trans*-3-haloacrylates to yield malonate semialdehyde by a mechanism utilizing Pro-1, Arg-8, Arg-11, and Glu-52. These residues are implicated in a promiscuous hydratase activity where 2-oxo-3-pentynoate is processed to acetopyruvate. The roles of three nearby residues (Asn-39, Phe-39, and Phe-50) are unexplored. Mutants were constructed at these positions (N39A, F39A, F39T, F50A and F50Y) and kinetic parameters determined along with those of the R8K and R11K mutants. Analysis indicates that Arg-8, Arg-11, and Asn-39 are critical for dehalogenase activity whereas Arg-11 and Phe-50 are critical for hydratase activity. Docking studies suggest structural bases for these observations.

1. Introduction

trans-3-Chloroacrylic acid dehalogenase (CaaD) is a heterohexameric enzyme, which consists of small α -subunits (75 amino acids) and β -subunits (70 amino acids), that converts the *trans*-isomers of 3-haloacrylates (**1** and **2**, Scheme 1) to malonate semialdehyde (**5**), presumably through a halohydrin or enol intermediate (**3** and **4**, respectively) [1,2]. CaaD is part of a catabolic pathway in *Pseudomonas pavonaceae* 170 for *trans*-1,3-dichloropropene, one of the active ingredients in the agricultural nematocides Shell D-D and Telone II [1,3,4]. The enzyme belongs to the tautomerase superfamily, a group of structurally homologous proteins characterized by a conserved α - β structural motif and a catalytically important amino-terminal proline [5,6].

In addition to the dehalogenation of the *trans*-3-haloacrylates, CaaD catalyzes the hydration of 2-oxo-3-pentynoate (**6**, Scheme 2A) and 3-chloro- and 3-bromopropiolate (**7** and **8** in Scheme 2B) [7]. Upon hydration, the 3-halopropiolates are converted into potent irreversible inhibitors of CaaD (an acyl halide or a ketene). Inactivation of the enzyme results from the covalent modification of Pro-1 (**9**, Scheme 2B). Hydration of **6** affords acetopyruvate (**10**), which does not inactivate the enzyme. Although not physiologically relevant, these reactions

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are analogous to the hydrolytic dehalogenation of **1** and **2**, and their study has provided much insight into the catalytic mechanism of CaaD [8].

For example, much knowledge has been gained from the crystal structure of CaaD inactivated by 3-bromopropiolate (**8** to **9**, Scheme 2B) [9]. The structure identified the active site and suggested interactions that might be responsible for binding and catalysis. In the structure of the inactivated enzyme (Figure 1, PDB entry 1S0Y), Pro-1 forms a covalent bond to the C-3 of a 3-oxopropionate moiety, which is the adduct resulting from the enzyme-catalyzed hydration of **8** (Scheme 2B). The carboxylate group interacts with Arg-8 and Arg-11, and the remaining portion of the adduct makes hydrophobic contacts with Phe-50, Leu-57, and Ile-37. The carboxylate group of Glu-52 appears fixed by the amide side chain of Asn-39. Phe-39 and Phe-50 contribute to the active site environment and form part of the active site wall.

Based on this arrangement of active site residues, a working hypothesis for the mechanism of CaaD was formulated (Scheme 3). The positions of Glu-52 and Pro-1 in the active site, coupled with the determination of a pK_a value for Pro-1 of ~9.3 [10], implicated Glu-52 as the general base catalyst that activates a water molecule for attack at C-3 of **1** (or **2**) and Pro-1 as the general acid catalyst that provides a proton at C-2. Mutagenesis of these residues confirmed their importance in the mechanism: there was no detectable activity for the E52Q mutant and greatly reduced activity for the P1A mutant [1,9]. The two arginines (Arg-8 and Arg-11) likely interact with the C-1 carboxylate group to align the substrate and draw electron density away from C-3. Such an interaction would make C-3 partially positively charged and facilitate the Michael addition of water to generate the intermediate (**3** or **4**) by route A or B (Scheme 3). Replacing either residue by alanine resulted in no detectable activity [1,9].

In this work, the contributions made by Asn-39, Phe-39, and Phe-50 to the activities of CaaD were assessed by mutagenesis and docking studies. Accordingly, Asn-39 was replaced with an alanine, Phe-39 was replaced with an alanine or a threonine, and Phe-50 was replaced with an alanine or a tyrosine. Subsequently, the kinetic parameters for each mutant-catalyzed reaction (using **1**, **2**, or **6**), as well as the pH-rate profile of the F50A-CaaD-catalyzed reaction (using **2**), were determined and compared to those of the wild-type. In addition, the roles of Arg-8 and Arg-11 were examined in more detail by analyzing the kinetic properties of both their alanine (with **6**) and lysine mutants (with **1**, **2**, and **6**). The results show that the interactions of Arg-8 and Arg-11, or a similar cationic group such as lysine, with substrate play an important role in both substrate binding and catalysis. The interaction between the amide side chain of Asn-39 and the carboxylate group of Glu-52 is critical for positioning the Glu-52 side chain in a favorable orientation for the activation of a water molecule [11,12]. This activation of water is essential for the conversion of the 3-haloacrylates **1** and **2**, but not for the conversion of the more electrophilic substrate **6**. Finally, the results suggest that Phe-50 plays a much more significant role in the CaaD reactions than Phe-39: Phe-50 is likely critical for maintaining the active site structural integrity and environment.

2. Material and methods

2.1. Materials

Chemicals, biochemicals, buffers, solvents, and molecular biology reagents were obtained from sources reported elsewhere [13], or as indicated below. Literature procedures were used for the synthesis of *trans*-3-bromoacrylic acid (**2**) and 2-oxo-3-pentynoate (**6**) [7,14]. The sources for the components of Luria-Bertani (LB) media as well as the enzymes and reagents used in the molecular biology procedures are reported elsewhere [13,15]. The

Amicon concentrator and the YM10 ultrafiltration membranes were obtained from Millipore Corp. (Billerica, MA). Pre-packed PD-10 Sephadex G-25 columns were purchased from Biosciences AB (Uppsala, Sweden). Oligonucleotides for DNA amplification and sequencing were synthesized by Genosys (The Woodlands, TX). *Escherichia coli* strain BL21-Gold(DE3) was obtained from Agilent Technologies (Santa Clara, CA). The construction of the R8A, R11A, R11K, and F39A mutants of CaaD are described elsewhere [1,9].

2.2. General Methods

Techniques for restriction enzyme digestion, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere [15]. The PCR was carried out in a Perkin-Elmer DNA thermocycler Model 480 obtained from Perkin Elmer Inc. (Wellesley, MA). DNA sequencing was performed by the DNA Core Facility housed in the Institute for Cellular and Molecular Biology (ICMB) at The University of Texas at Austin. Mass spectral data was collected in the ICMB Protein and Metabolite Facility. Samples were made up as previously described [7]. HPLC was performed on a Waters (Milford, MA) 501/510 system using either a TSKgel DEAE-5PW (anion exchange) or a TSKgel Phenyl-5PW (hydrophobic interaction) column (Tosoh Bioscience, Montgomeryville, PA). Protein was analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions using sodium dodecyl sulfate (SDS) on gels containing 15% polyacrylamide [16]. The gels were stained with Coomassie brilliant blue. Protein concentrations were determined using the Waddell method [17]. The native molecular masses of CaaD and the mutant enzymes were determined by gel filtration chromatography on a Superose 12 column (Pharmacia Biotech AB, Uppsala, Sweden) using the Waters 501/510 HPLC system. Kinetic data were obtained on a Hewlett Packard 8452A or an Agilent 8453 Diode Array spectrophotometer. The contents of 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.

2.3. Construction of the CaaD Mutants

The five CaaD mutants were constructed using the coding sequence for CaaD in pET44T2 as the template [1]. The R8K mutant was generated by the PCR using the primer 5 - **CACGGCATATGCCGATGATCTCTTGC**GACATGAAATATGGGAGAACA-3, in which the *NdeI* restriction site is shown in bold and the codon introducing the mutation is underlined. This primer corresponds to the 5' end of the wild-type coding sequence and was used in combination with the reverse primer (primer R), 5' - TTGCCCAAGCAGAGGGATCCCCTAGCT-3, in which the *BamHI* site is shown in bold. The F39T, F50A, F50Y, and N39A mutants were generated by overlap extension PCR [18]. The forward primer (primer F), 5' - **CACGGCATATGCCGATGATCTCTTGC**GAC-3, where the *NdeI* site is shown in bold, and primer R (above) were used as the external primers. The internal primers were as follows: 5' -CCCCGCGAGAACATTACCTTTGTGATTTCGG-3 and 5' - CCGAATCACAAGGTAATGTTCTCGCGGGG-3 for the F39T mutant; 5' - ATTCGGGAGGGCAGCGGGATCAACGCCGTTGAGCACGGC-3 and 5' - GTTGATCCCGCTGCCCTCCCGAAT-3 (primer A) for the F50A mutant; 5' - ATTCGGGAGGGCAGCGGGATCAACTACGTTGAGCACGGC-3 and primer A for the F50Y mutant; and 5' -TCCGACCCCAAGATCATCGCTGTGCTTCTGGTG-3 and 5' - GATGATCTTGGGGTCGGA-3 for the N39A mutant. The codon introducing the mutation is underlined in each primer. The PCR reactions were carried out as described before, and the products were purified and cloned into the pET3a vector (Promega Corp.,

Madison, WI) for expression of the mutant genes [1]. The mutant genes were sequenced in order to verify that only the intended changes had been introduced.

2.4. Expression, Purification, and Characterization of the CaaD Mutants

Expression and purification of the CaaD mutants followed previously described procedures [1,7]. The purified enzymes were analyzed by electrospray ionization mass spectrometry (ESI-MS). The specific dehalogenase activities of the purified mutant enzymes were measured by incubating an appropriate amount of enzyme with 3 mL of 10 mM substrate (either **1** or **2**) in 50 mM Tris-SO₄ buffer (pH 8.2) at 22°C. Halide release was monitored colorimetrically as described before [1].

Kinetic assays were performed in 20 mM sodium phosphate buffer (pH 9.0) at 22 °C. The hydration of the *trans*-3-haloacrylates was monitored by following the decrease in absorbance at 224 nm, which corresponds to the hydration of **1** ($\epsilon = 4900 \text{ M}^{-1}\text{cm}^{-1}$) and **2** ($\epsilon = 9700 \text{ M}^{-1}\text{cm}^{-1}$) [7]. The hydration of **6** was monitored by following acetopyruvate (**10**) production at 294 nm ($\epsilon = 7000 \text{ M}^{-1}\text{cm}^{-1}$) [7]. The pH dependence of the steady-state kinetic parameters was measured in 20 mM sodium phosphate buffers, with pH values ranging from 6.0–9.1, as previously described [10].

2.5. Docking Studies

Docking studies were carried out using PyMOL with Autodock Vina using **2** and **6** [19,20]. The strategy for the identification of a specific active site to use in the docking studies is described elsewhere [12]. A CaaD active site with the 3-oxopropanoate moiety covalently bound to Pro-1 (PDB entry 1S0Y) was selected as the receptor site for docking studies. The atoms making up this adduct were removed (*in silico*) before the docking experiments were carried out. Docking trials were restricted to a $15\text{\AA} \times 15\text{\AA} \times 15\text{\AA}$ box centered on the Pro-1 residue. The R8K, R11K, and F50A mutants of CaaD were individually designed *in silico* using Pymol mutagenesis wizard [21]. The selected lysine rotomers had minimal steric clashes and placed the positively charged amino group nearest to the location observed for the guanidinium moieties of the arginines in the wild type enzyme. The side chains of E52, R8K and R11K were made allowed to rotate freely in their individual docking routines.

3. Results

3.1. Expression, Purification, and Characterization of the CaaD Mutants

Five single site-directed mutants (R8K, F39T, N39A, F50A, and F50Y) of CaaD, constructed in this work, and the four previously constructed mutants (R8A, R11A, R11K, and F39A) were overexpressed in *E. coli* strain BL21(DE3), and purified to >95% homogeneity (as assessed by SDS-PAGE) using previously described procedures [1,7,9]. DNA sequencing verified that only the intended mutations had been introduced into each mutant gene. The yields (in mg of homogeneous protein per liter of cell culture) of the mutants varied from 5–25 mg.

The nine purified CaaD mutants were analyzed by ESI-MS and gel filtration chromatography. Mass spectral analysis of the individual mutants showed two major peaks corresponding to the expected molecular masses of the α - and β -subunit of each mutant (Table 1). This observation indicates that each mutant has undergone posttranslational processing to remove the initiating methionine, resulting in α - and β -subunit with an N-terminal proline [22,23]. In addition, the experimentally obtained molecular masses of the purified mutant proteins confirmed the presence of only the intended amino acid substitutions. The mutant enzymes migrated during gel filtration chromatography comparably with the wild-type enzyme, suggesting that gross conformational changes are

not likely present and that the characteristic heterohexameric quaternary structure of the wild-type is present in the mutant enzymes [9].

3.2. Specific Activities of the Mutant Enzymes

Specific activities for the nine mutant enzymes were measured using saturating concentrations of the *trans*-3-haloacrylates and expressed relative to the wild-type enzyme (Table 2) [1]. Changing Arg-8 to an alanine or a lysine and changing Arg-11 to an alanine (individually) essentially abolished catalytic activity of CaaD, confirming that these arginines are critical for enzymatic activity. The R11K mutant had partially restored catalytic activity compared to the catalytically inactive R11A mutant, suggesting that a positive charge at this position is important for activity. The F39A, F39T, N39A, and F50A mutants are also able to process both *trans*-3-haloacrylates, although with reduced activities (Table 2). In contrast, changing Phe-50 to a tyrosine results in an enzyme with increased activity (~2.3-fold) for both *trans*-3-haloacrylates.

3.3 Kinetic Analysis of the Mutant Enzymes

The steady-state kinetic parameters for the hydratase activity of the nine mutants were measured using the *trans*-3-haloacrylates (**1** and **2**) (Table 3) and 2-oxo-3-pentynoate (**6**) (Table 4) as substrates, and compared to the kinetic parameters for the hydratase activity of wild-type CaaD. Changing Arg-8 to an alanine or a lysine results in enzymes with no measurable activity using **1** or **2**. These mutants, however, are still able to process **6** to **10**, although at reduced catalytic efficiencies. For the R8A mutation, there is a ~14-fold decrease in k_{cat} and a ~3.2-fold increase in K_{m} . As a result, the $k_{\text{cat}}/K_{\text{m}}$ is reduced ~46-fold. For the R8K mutation, there is a ~12-fold decrease in k_{cat} and a ~7.3-fold decrease in K_{m} . This results in a $k_{\text{cat}}/K_{\text{m}}$ that is nearly comparable to that of wild-type. Changing Arg-11 to an alanine results in an enzyme with no detectable activity using any of the three substrates. However, the R11K CaaD is an active enzyme. Using **1**, the k_{cat} for the R11K-catalyzed reaction is estimated to be down at least 47-fold, but an accurate K_{m} value could not be determined. Using **2**, the k_{cat} for the R11K-catalyzed reaction is down ~39-fold and the K_{m} is up ~6.7-fold, resulting in a ~264-fold reduction in $k_{\text{cat}}/K_{\text{m}}$. Using **6**, the k_{cat} for the R11K-catalyzed reaction is up ~2.4-fold and the K_{m} is up ~15-fold, resulting in a ~6.4-fold decrease in $k_{\text{cat}}/K_{\text{m}}$.

Changing N39 to an alanine yields an enzyme with very low-level activity using **1** and **2**, precluding the accurate determination of kinetic parameters for these reactions. However, this mutation has little effect on the kinetic parameters for the hydratase activity using **6**. The k_{cat} for the N39A-catalyzed reaction is down ~3.5-fold and the K_{m} is down ~2.8-fold, resulting in a nearly comparable $k_{\text{cat}}/K_{\text{m}}$ to that of wild-type.

In contrast to the large effect of mutations at positions Arg-8 and Arg-11 on the catalytic efficiency of CaaD, the mutation of Phe-39 to either a threonine or an alanine has less drastic effects on k_{cat} and K_{m} . Using **1**, the k_{cat} for the F39A-catalyzed reaction is down ~8.3 fold and the K_{m} is down ~1.5-fold, resulting in a ~5.5-fold decrease in $k_{\text{cat}}/K_{\text{m}}$. For the F39T mutant, there is a ~1.9-fold decrease in k_{cat} and a ~3.1-fold increase in K_{m} using **1**. As a result, the $k_{\text{cat}}/K_{\text{m}}$ is reduced ~5.7-fold. Using **2**, the k_{cat} for the F39A-catalyzed reaction is down ~11.6-fold and the K_{m} is down ~2-fold, resulting in a ~5.8-fold decrease in $k_{\text{cat}}/K_{\text{m}}$. For the F39T mutant, there is a ~2.5-fold decrease in k_{cat} and a ~1.2-fold increase in K_{m} using **2**. As a result, the $k_{\text{cat}}/K_{\text{m}}$ is reduced ~3.1-fold. Using **6**, the k_{cat} for the F39A-catalyzed reaction is down ~3.5 fold and the K_{m} is down ~4.4-fold, resulting in a nearly comparable $k_{\text{cat}}/K_{\text{m}}$ to that of wild-type. For the F39T mutant, there is a ~2.3-fold decrease in k_{cat} and a ~10-fold decrease in K_{m} using **6**. As a result, the $k_{\text{cat}}/K_{\text{m}}$ is increased ~4.2-fold.

Changing Phe-50 to an alanine has a substantial effect on both k_{cat} and K_{m} . For the F50A mutant, there is a ~20-fold and a ~18.2-fold decrease in k_{cat} , and a ~9-fold and a ~4.6-fold increase in K_{m} using **1** and **2**, respectively. As a result, the $k_{\text{cat}}/K_{\text{m}}$ is reduced ~176-fold (using **1**) or ~87-fold (using **2**). Interestingly, the reaction of the F50A mutant with **6** follows a biphasic time course, consisting of an initial burst of acetopyruvate (**10**) formation (equal to one equivalent of enzyme) followed by a phase that corresponds to steady state formation of **10** (data not shown). At first glance, this observation could be indicative that product release is the rate-limiting step in the reaction. The kinetics and mechanism of the F50A-catalyzed hydration of **6** are under investigation.

In contrast to the decreased catalytic activity of the F50A mutant, changing Phe-50 to a tyrosine has a positive effect on k_{cat} . For the F50Y mutant, there are ~2.5-fold and ~2.9-fold increases in k_{cat} , and ~3.9-fold and ~3.2-fold increases in K_{m} , using **1** and **2**, respectively. As a result, the $k_{\text{cat}}/K_{\text{m}}$ is reduced slightly (~1.5-fold using **1** and ~1.1-fold using **2**). Using **6**, the F50Y mutation has no effect on k_{cat} and only little effect on K_{m} (~2.4-fold decrease). This results in a ~2.4-fold increase in $k_{\text{cat}}/K_{\text{m}}$.

3.4. pH Dependence of the Kinetic Parameters of α F50A

The pH dependences of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ for the F50A-catalyzed hydration of **2** were determined in 20 mM sodium phosphate buffer over the pH range 6.0–9.1 [10]. The pH dependences of k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ are given by the equations:

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

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

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 $k_{\text{cat}}/K_{\text{m}}$ is the $k_{\text{cat}}/K_{\text{m}}$ of the enzyme in the single protonated form [24,25].

A plot of $\log k_{\text{cat}}/K_{\text{m}}$ versus pH shows a bell-shaped dependence with limiting slopes of unity on either side of the pH maximum, consistent with eq 2, indicating that both a basic group and an acidic group on the enzyme are important for catalysis, where only the single protonated form of the enzyme is active [24]. The substrate ($\text{p}K_{\text{a}} = 4.6$) does not titrate in the pH range studied. A fit of the pH-dependence of $k_{\text{cat}}/K_{\text{m}}$, which follows the ionization of the free enzyme, to eq (2) gives $\text{p}K_{\text{a}}$ values for the free enzyme of $\text{p}K_1 = 6.5 \pm 0.3$ and $\text{p}K_2 = 7.9 \pm 0.2$ (data not shown). Significantly different values ($\text{p}K_1 = 7.6 \pm 0.2$ and $\text{p}K_2 = 9.2 \pm 0.2$) were previously reported for the wild-type enzyme.

At saturating levels of the substrate **2**, the pH dependence of k_{cat} shows that the $\text{p}K_{\text{a}}$ of the basic group is undetectable, suggesting it has decreased below 6.0. This behavior has also been reported for the wild type CaaD [10], and can be fit by eq (1), but in the present case $K_1 [H^+]$ causing the middle term in the denominator to disappear. A fit of the pH-dependence of k_{cat} , which follows the ionization of the enzyme-substrate complex, to the modified eq (1) yields a $\text{p}K_2$ value for the enzyme-substrate complex of $\text{p}K_{\text{a}} = 7.9 \pm 0.1$ (data not shown). This value also differs significantly from that previously reported for the hydratase activity for the wild-type enzyme using **2** ($\text{p}K_2 = 9.5 \pm 0.1$).

3.5 In silico Docking Experiments Using **2**

In order to provide insight into the kinetic results, docking studies were carried out with CaaD using **2**. The docking experiment with **2** (Figure 2) is an extension of a previously

published docking study [12]. This study suggested that Glu-52 might play roles in both substrate binding and product release in addition to participating in the chemistry (i.e., activating water for attack). In all crystal structures of CaaD, Glu-52 is observed in two positions [9,11]. In one position, the carboxylate moiety is rotated out of the active site (PDB entry 3EJ3). In the other position, the carboxylate moiety points directly into the active site (PDB entry 1S0Y). Rotating between these two positions could play a role in product release and, as such, possibly limit the rate of turnover. The results of the docking studies with **2** also show the two positions. The two best predicted scores place the carboxylate moiety (of Glu-52) out of, or into the active site (Figures 2A and B). In the crystal structures of CaaD, Asn-39 is within hydrogen bonding distance of Glu-52. Regardless of the position of Glu-52 (out of, or into the active site), the carboxylate moiety of Glu-52 remains within hydrogen bonding distance of the amide moiety of Asn-39 (dotted lines in Figures 2A and B). The N39A mutant of CaaD eliminates the interaction, which is consistent with the significantly reduced activity for the *trans*-3-haloacrylates (even though Asn-39 is more than 5 Å away from the prolyl nitrogen of Pro-1 and C3 of **2**).

Docking studies were also carried out with the individual *in silico* R8K and R11K mutants of CaaD and **2** (Figures 3 and 4). The results provide a possible explanation for the kinetic data collected for the R8K and R11K mutants of CaaD. When **2** is docked into the R8K mutant of CaaD, it is pulled toward the remaining Arg-11 residue (Figure 3), moving the C3 carbon away from the proposed site of water attack (near Glu-52) by about 1.2 Å. Moreover, the lysyl amino group in the K8 residue is never observed in a position where it can form an ion-pair or salt bridge with the carboxylate moiety of **2**. However, when **2** is docked into the R11K mutant of CaaD, **2** remains in the same position (within error) as that observed in CaaD (Figure 4). Similar to the results for the R8K mutant, the docking routines never place the lysyl amino group of the K11 residue within bonding distance of the carboxylate moiety such that it can form an ion-pair or salt bridge. It appears that the guanidinium moiety of Arg-8 is more important than that of Arg-11 in positioning **2** for the proposed chemistry. The experimental kinetic data agree with the docking results showing that the R11K mutant maintains 26% relative specific activity while the R8K mutant yields no detectable activity.

3.6 In silico Docking Experiments Using **6**

Docking studies were also carried out with CaaD and **6**, as well as two mutants of CaaD (R11K and F50A). Docking **6** into the active site of CaaD predicts a similar binding orientation to that observed for **2**. However, there are two equivalent predicted binding orientations for **6** (equivalent based on docking scores), both showing five hydrogen bonds to **6**: two to each of the active site arginines (Arg-8 and Arg-11) and one to the backbone amide nitrogen of Arg-8 (Figures 5A and B). Both place the C4 of **6** in the same general location as the C3 group of **2** (proximal to Glu-52). The major difference between the two orientations is the potential hydrogen bonding partners of the C2 carbonyl group of **6**. One orientation places the C2 carbonyl group facing the back of the pocket where it can hydrogen bond to the backbone amide of Arg-8 (Figure 5A). The second binding orientation places the C2 carbonyl group facing the front of the pocket with a predicted hydrogen bond to the guanidinium group of Arg-8 (Figure 5B). The residual activity observed for the R8A and R8K mutants of CaaD suggests that the first binding orientation might be preferred. Polarization of the unsaturated system through the C2 carbonyl group (drawing electron density away from C4) is thought to be important for the reactivity of **6**. In the first orientation, the additional hydrogen bonding interaction is maintained with the backbone amide of the residue at position 8 (Arg, Ala, or Lys).

The docking results of **6** with the *in silico* R11K mutant of CaaD show no qualitative difference in the location of **6** between wild type and the R11K mutant. However, the R11K mutant of CaaD is predicted to have a higher free energy of binding, but not high enough to account for the difference in K_m (10-fold increase in Table 4 vs. a predicted 2-fold increase, as calculated by the ΔG of binding). Although the K_m is probably higher for the R11K mutant of CaaD, the one reported in Table 4 might be an artifact of non-saturating conditions.

The docking results of **6** with the *in silico* F50A mutant of CaaD illustrates the importance of a bulky side chain group in this position to maintain the integrity of the active site pocket. When Phe-50 is changed to an alanine residue (*in silico*), the pocket volume increases dramatically and a tunnel forms that leads all the way into the core of the trimer. This extra space is utilized by the docking routine, which attempts to bury and twist **6** into the newly available space (Figure 6). This orientation positions the C4 carbon of **6** a significant distance away from the Glu-52 residue. (The docking results include orientations comparable to that observed for the wild type, but they have lower docking scores.) The docking results of **2** with the *in silico* F50A mutant of CaaD show a similar orientation to that observed for **6** (not shown), which could account for the diminished activity.

4. Discussion

The current working hypothesis for the mechanism of CaaD (Scheme 3) is based on the cumulative results of sequence analysis [1], studies using intermediate analogues as well as reversible and irreversible inhibitors [2,7], site-directed mutagenesis [1,7,9,10], NMR experiments [10], pre-steady-state kinetic analysis [12], and crystallographic studies [9,11]. The importance of Pro-1 and Arg-11 in the hydrolytic dehalogenation of **1** and **2** was initially established by sequence analysis and site-directed mutagenesis [1]. The same study determined that the R11K mutant partially restores activity and that the F39A mutant slows the reaction, such that Phe-39 is not essential for activity [1]. The bulk of the experimental evidence supporting the mechanism came from inhibition as well as NMR and crystallographic studies [7,9,10]. These studies identified the promiscuous hydratase activity of CaaD (i.e., **6** to **10** in Scheme 2A) and the irreversible inhibition of CaaD by **7** and **8** (Scheme 2B) [7]. The results led to the crystal structure of the inactivated CaaD, which identified Arg-8 and Glu-52 as the two additional players in the mechanism [9]. Subsequent site-directed mutagenesis confirmed their importance. In addition to the position of Glu-52 in the crystal structure, a hydrogen bond was observed between Glu-52 and the backbone carbonyl group of Ile-37. This observation indicated that side chain of Glu-52 is a free acid, which is attributed to the abstraction of a proton from water to produce the inactivating species (either acyl halide or ketene in Scheme 2B). Hence, Glu-52 is proposed to function as the water-activating base. The NMR studies determined a pK_a of 9.3 for Pro-1, suggesting it can be a general acid catalyst and provide a proton at C-2 [10]. Finally, CaaD shows a robust phenylpyruvate tautomerase activity, converting phenylenolpyruvate (**11**, Scheme 4) to phenylpyruvate (**12**). This observation is consistent with an enol intermediate (**4**, Scheme 3 route B) in the reaction, although a halohydrin intermediate cannot be ruled out [2].

The crystal structure of the inactivated CaaD [9] along with a more recent structure of the enzyme in a complex with acetate (from the precipitant buffer) [11] prompted an examination of the roles of Asn-39, Phe-39, and Phe-50 in the dehalogenation mechanism as well as a more extensive analysis of the roles of Arg-8 and Arg-11.

Asn-39, Arg-8, and Arg-11 are absolutely essential for activity (although the R11K mutant is partially active using **2**). Phe-50 is important for activity, but not as critical as

Asn-39, Arg-8, and Arg-11. Phe-39 is the least critical residue in this group, as previously reported [1].

The crystal structure of CaaD (where acetate is the ligand) shows a hydrogen bond between the amide side chain of Asn-39 and the carboxylic acid side chain of Glu-52 [11]. An overlay of multiple active sites from multiple structures shows the side chain of Glu-52 in two conformations (out of, and into the active site, Figures 2A and B) [9,11]. However, the hydrogen bond between the side chains of Asn-39 and Glu-52 is maintained. These observations prompted the suggestion that Asn-39 performs a critical role in switching Glu-52 between conformations where it activates water in one conformation and enables the release of product in the other conformation [11,12]. Switching between conformations could also facilitate the movement of water molecules in and out of the active site for the dehalogenation reaction [11]. The switch between conformations might be the unknown partially rate limiting step, identified by a recent pre-steady state kinetic analysis of the reaction [12].

Like Asn-39, Arg-8 and Arg-11 are critical for dehalogenation activity. The combined modeling and mutagenesis results provide two new pieces of information. First, the guanidinium group of Arg-8 is more important for activity than that of Arg-11 because it positions C-3 of substrate for attack by water. Docking **2** into the active site of the R8K mutant moves C3 away from the site of water attack (~1.2 Å) toward the remaining Arg-11 (Figure 3). Second, although the R11K mutant partially restores activity, the side chain of lysine seems to be too flexible for efficient catalysis. The modeling studies suggest the amino group is not able to interact with the substrate's C1 carboxylate group in the R8K or R11K mutants.

Phe-50 is important for activity, but not as important as Asn-39, Arg-8, and Arg-11. This residue likely maintains the structural integrity of the active site, and the loss of the phenyl side chain has significant consequences for activity and the pK_a values of active site residues. This might be due to a combination of the newly created space and the breach of the active site wall. It is interesting to note that replacing Phe-50 with a tyrosine results in a more active dehalogenase, mostly due to the increase in k_{cat} . The basis for the increased activity is not known. Phe-39 is less important for dehalogenation activity although it along with Ile-37, Leu-57, and Phe-50 comprises the halide-binding pocket. Evidently, Phe-39 is not an important component of this pocket or the pocket is not an important part of the catalytic strategy.

2-Oxo-3-pentynoate (**6**) has served as a highly informative mechanistic probe of tautomerase superfamily members. It was first synthesized and used as an active-site-directed irreversible inhibitor of 4-oxalocrotonate tautomerase (4-OT) [14,26]. The crystal structure of the inactivated enzyme identified the active site groups involved in inactivation and suggested a mechanism for inactivation as well as the biological reaction [26]. Subsequently, the acetylene compound was used as a probe of the CaaD-catalyzed reaction as well as four additional reactions, those catalyzed by *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD) [27], malonate semialdehyde decarboxylase (MSAD) [28], and two *cis*-CaaD homologues, one from *Corynebacterium glutamicum* designated Cg10062 [29], and one from *Mycobacterium smegmatis* designated MsCCH2 [30]. *cis*-CaaD, MASD and Cg10062 convert **6** to **10**, whereas MsCCH2 catalyzes a hydration reaction at low pH, but is inactivated at higher pH (*vide infra*). These different reactions are attributed to the different pK_a values of Pro-1. The hydration reaction predominates when Pro-1 is cationic and has a higher pK_a (CaaD, *cis*-CaaD, MSAD, MsCCH2 below its pK_a), whereas inactivation is the primary reaction when Pro-1 is neutral due to a lower pK_a (4-OT and MsCCH2 above its pK_a).

The mechanism for the enzyme-catalyzed conversions of **6** to **10** has not been examined in detail, but has always been thought to involve the Michael addition of water to **6** (Scheme 5) [27–30]. In this mechanism for the CaaD-catalyzed reaction, Glu-52 activates a water molecule for nucleophilic attack at C-4 of **6** while Arg-8 and Arg-11 polarize the carbonyl oxygen and assist in binding of the carboxylate group. Specific roles for Arg-8 and Arg-11 in the mechanism have not been assigned. Pro-1 provides a proton at C-3 to complete the Michael addition of water. (The ketonization of **13** to **10** in Scheme 5 could be an enzyme-catalyzed process or result from a non-enzymatic rearrangement.)

The mutagenesis and modeling results reported here provide further insight into the hydration reaction. Both Arg-11 and Phe-50 are critical for hydratase activity. In contrast to the dehalogenation reaction, changing Asn-39 to an alanine has little effect on the hydration reaction. Like the dehalogenation reaction, changing Phe-39 has little effect on the hydration reaction. Modeling suggests that Arg-11 and Arg-8 are important for binding (via the C1 carboxylate group) and that the backbone amide of Arg-8 is important for polarization of the π -unsaturated system (via the C2 carbonyl group). These observations suggest that the positioning of **6** in the active site in the correct (or a fortuitous) orientation for water addition might be more important than activating water or activating the substrate for a reaction.

The behavior of the *cis*-CaaD homologue designated MsCCH2 with **6** might explain, at least in part, the behavior of the F50A-catalyzed reaction with **6**. Baas et al. reported that incubation of MsCCH2 with **6** results in the formation of **10** as well as the inactivation of the enzyme by covalent modification of Pro-1 [30]. Moreover, the partitioning of **6** between these two processes is pH dependent. At pH 6.5, the enzyme is predominantly a hydratase (reflecting a cationic Pro-1). At pH 8.5, the enzyme is inactivated (reflecting a neutral Pro-1). At pH 7.3–7.5, the enzyme partitions between hydration and inactivation.

The reaction of the F50A-catalyzed reaction with **6** has not been examined in detail. However, the removal of the side chain of Phe-50 has serious consequences for the active site, as evidenced by the sharp decrease of the pK_a values for both the base and acid catalysts (to 6.5 from 7.6 and 7.9 from 9.2, respectively). The observation that there is a burst of production of **10** followed by its steady state production might involve a partitioning between these two processes, or some other process. Another possibility is that product release is now rate-limiting, as is observed with the CaaD-catalyzed turnover of **2** [12]. The kinetics and mechanism of the F50A-catalyzed reaction with **6** are under investigation.

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Fig. 1.

A close-up of one CaaD active site where the prolyl nitrogen is covalently attached to the 3-oxopropanoate moiety (PDB entry 1S0Y). The residues comprising the active site include P1, I37, N39, E52, F39, L57, F50, R8, and R11 (clockwise from P1). The prime indicates that F39 is from an adjacent subunit. The core set of residues consists of P1, E52, R8, and R11. The residues under investigation nearby the core set are N39, F39, and F50. A putative halide binding pocket consists of I37, L57, F39, and F50. The Figure was created with PyMol [20].

Fig. 2.

The top two poses observed in the docking studies of CaaD with **2** (green stick). A) The "open conformation" pose where the carboxylate group of Glu-52 points out of the active site and increases the active site volume. B) In a second pose, the carboxylate group of Glu-52 is positioned to activate a water molecule. In both poses, the carboxylate group is within hydrogen bond distance of Asn-39 (shown by dotted lines). The open conformation pose has a slightly better docking score than the other pose. The Figure was created with PyMol [20].

Fig. 3.

The docking of **2** (blue line) in the *in silico* R8K mutant of CaaD. The interaction of the C1 carboxylate group of **2** with Arg-11 is shown in the mutant. This docking pose moves C3 of **2** away from Glu-52, possibly leading to the observed greatly reduced activity of R8K mutant (Table 3). For comparison, the docking pose for **2** (green line) in CaaD is shown (from Figure 3B) [12]. The C-3 positions of **2** are shown by the arrows. The Figure was created with PyMol [20].

Fig. 4.

The docking of **2** (pink line) in the *in silico* R11K mutant of CaaD. The binding pose is comparable to that observed for wild type with **2** (green line) (from Figure 3B) [12]. The comparable binding poses might be partially responsible for the observation that the R11K mutant of CaaD shows activity with **2**, although reduced relative to the wild type (Table 3). The C-3 positions of **2** are shown by the arrows. The Figure was prepared using PyMOL [20].

Fig. 5.

The two equivalent docking poses for **6** (teal stick) in the active site of CaaD. A) In this pose, five hydrogen bonds are shown between the C1 carboxylate and the C2 carbonyl group of **6** and CaaD: two hydrogen bonds are shown to each of the two arginines (Arg-8 and Arg-11) and one hydrogen bond is shown between the C2 carbonyl group and the backbone amide nitrogen of Arg-8. B) The second pose shows the same hydrogen bonds but the C2 carbonyl group interacts with the guanidinium group of Arg-8. The Figure was prepared using PyMOL [20].

Fig. 6. Compound **6** (salmon stick) docked in the F50A mutant of CaaD modeled *in silico*. The predicted cavities in the mutant are shown in salmon transparent shading. The cavity behind Ala-50 forms a tunnel to the interior of the enzyme. This space is occupied by Phe-50 in the wild-type enzyme. The docking routine twists **6** ~90° from the position shown in Figure 5A, burying C5, C4, and C3 into the newly formed tunnel. The Figure was prepared using PyMOL [20].

Scheme 1.

Scheme 2.

Scheme 3.

Scheme 4.

Scheme 5.

Table 1

The Observed and Calculated Monomer Masses of Wild-type CaaD and the Active Site Mutants

Enzyme	Observed mass of the α -subunit ^a	Calculated mass of the α -subunit ^b	Observed mass of the β -subunit ^a	Calculated mass of the β -subunit ^b
CaaD	8,343	8,343	7,506	7,506
R8A	8,257	8,258	7,506	7,506
R8K	8,314	8,315	7,505	7,506
R11A	8,257	8,258	7,506	7,506
R11K	8,313	8,315	7,505	7,506
F39A	8,266	8,267	7,506	7,506
F39T	8,297	8,297	7,505	7,506
N39A	8,343	8,343	7,463	7,463
F50A	8,266	8,267	7,505	7,506
F50Y	8,359	8,359	7,506	7,506

^aThe masses of the two subunits of each of the purified proteins were determined by electrospray ionization mass spectrometry [7]. A sample generates two major peaks in the mass spectrum (after deconvolution) that correspond to the expected masses of the α - and β -subunits. The observed masses of the two peaks indicate that the amino-terminal proline is not blocked in either subunit by the initiating methionine [22,23].

^bThe monomer masses are predicted from analysis of the translated amino acid sequences of the two genes coding for the α - and β -subunits of CaaD, but without the initiating methionines.

Table 2Specific Activities of Wild-type CaaD and the Active-site Mutants^a

Enzyme	Relative specific activity with 1	Relative specific activity with 2
CaaD	100	100
R8A	< 0.01	< 0.01
R8K	< 0.01	< 0.01
R11A	< 0.01	< 0.01
R11K	19	26
F39A	20	9
F39T	31	25
N39A	3	5
F50A	25	5
F50Y	233	225

^aThe specific activities were determined in 50 mM Tris-SO₄ buffer (pH 8.2) at 22°C using saturating concentrations (i.e., 10 mM) of the indicated substrate [1]. Specific activity data are expressed relative to wild-type CaaD values, which are defined as 100 and are equivalent to 5080 mU/mg and 12210 mU/mg for *trans*-3-chloroacrylate (**1**) and *trans*-3-bromoacrylate (**2**), respectively, where 1 mU/mg is defined as 1 nmol of halide produced per min per mg of protein. The error associated with specific activity measurements is less than 10% of the value.

Table 3Kinetic Parameters for the Hydration of **1** and **2** by CaaD and the Active Site mutants^a

enzyme	substrate	k_{cat} (s ⁻¹)	K_{m} (μM)	$K_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
CaaD ^b	1	3.8 ± 0.1	31 ± 2	1.2 × 10 ⁵
	2	5.1 ± 0.1	37 ± 2	1.4 × 10 ⁵
R8A	1	< 1.5 × 10 ⁻³	-	-
	2	< 1.5 × 10 ⁻³	-	-
R8K	1	< 1.5 × 10 ⁻³	-	-
	2	< 1.5 × 10 ⁻³	-	-
R11A	1	< 1.5 × 10 ⁻³	-	-
	2	< 1.5 × 10 ⁻³	-	-
R11K	1	0.08 ± 0.004 ^c	-	-
	2	0.13 ± 0.01	247 ± 41	5.3 × 10 ²
F39A	1	0.46 ± 0.02	21 ± 3	2.2 × 10 ⁴
	2	0.44 ± 0.02	18 ± 3	2.4 × 10 ⁴
F39T	1	2.0 ± 0.1	97 ± 8	2.1 × 10 ⁴
	2	2.0 ± 0.1	44 ± 3	4.5 × 10 ⁴
N39A	1	< 1.5 × 10 ⁻²	-	-
	2	< 1.5 × 10 ⁻²	-	-
F50A	1	0.19 ± 0.03	280 ± 75	6.8 × 10 ²
	2	0.28 ± 0.03	170 ± 33	1.6 × 10 ³
F50Y	1	9.5 ± 0.5	120 ± 11	7.9 × 10 ⁴
	2	14.9 ± 1.1	118 ± 17	1.3 × 10 ⁵

^aThe steady-state kinetic parameters were determined in 20 mM sodium phosphate buffer (pH 9.0) at 22 °C. Errors are standard deviations.^bThese kinetic parameters were taken from Wang et al. [7].^cEstimated based on the measured specific activity using 10 mM of **1** (Table 2).

Table 4Kinetic Parameters for the Hydration of **6** by CaaD and the Active Site Mutants^a

enzyme	k_{cat} (s ⁻¹)	K_{m} (μM)	$K_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
CaaD ^b	0.7 ± 0.02	110 ± 4	6.4 × 10 ³
R8A	0.05 ± 0.01	350 ± 90	1.4 × 10 ²
R8K	0.06 ± 0.005	15 ± 2	4.0 × 10 ³
R11A	<0.01	-	-
R11K	1.7 ± 0.1	1640 ± 140	1.0 × 10 ³
F39A	0.2 ± 0.01	25 ± 3	8.0 × 10 ³
F39T	0.3 ± 0.01	11 ± 1	2.7 × 10 ⁴
N39A	0.2 ± 0.01	40 ± 6	5.0 × 10 ³
F50A	^c	-	-
F50Y	0.7 ± 0.02	45 ± 3	1.6 × 10 ⁴

^aThe steady-state kinetic parameters were determined in 20 mM sodium phosphate buffer (pH 9.0) at 22 °C. Errors are standard deviations.

^bThese kinetic parameters were taken from Wang et al. [7].

^cThe reaction of F50A-CaaD with **6** follows a biphasic time course, consisting of an initial burst of acetopyruvate (**10**) formation followed by a phase that corresponds to steady state formation of **10**. The kinetics and mechanism of this reaction are under investigation.