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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 βPro-1, αArg-8, αArg-11, and αGlu-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 (βAsn-39, αPhe-39, and αPhe-50) are unexplored. Mutants were constructed at these positions (βN39A, αF39A, αF39T, αF50A and αF50Y) and kinetic parameters determined along with those of the αR8K and αR11K mutants. Analysis indicates that αArg-8, αArg-11, and βAsn-39 are critical for dehalogenase activity whereas αArg-11 and αPhe-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 β–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 βPro-1 (**9**, Scheme 2B). Hydration of **6** affords acetopyruvate (**10**), which does not inactivate the enzyme. Although not physiologically relevant, these reactions 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 (Fig. 1, PDB entry 1S0Y), βPro-1 forms a covalent bond to the C-3 of a 3-oxopropanoate moiety, which is the adduct resulting from the enzyme-catalyzed hydration of **8** (Scheme 2B). The carboxylate group interacts with αArg-8 and αArg-11, and the remaining portion of the adduct makes hydrophobic contacts with αPhe-50, αLeu-57, and βIle-37. The carboxylate group of αGlu-52 appears fixed by the amide side chain of βAsn-39. αPhe-39 and αPhe-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 αGlu-52 and βPro-1 in the active site, coupled with the determination of a pK_a value for βPro-1 of ~9.3 [10], implicated αGlu-52 as the general base catalyst that activates a water molecule for attack at C-3 of **1** (or **2**) and βPro-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

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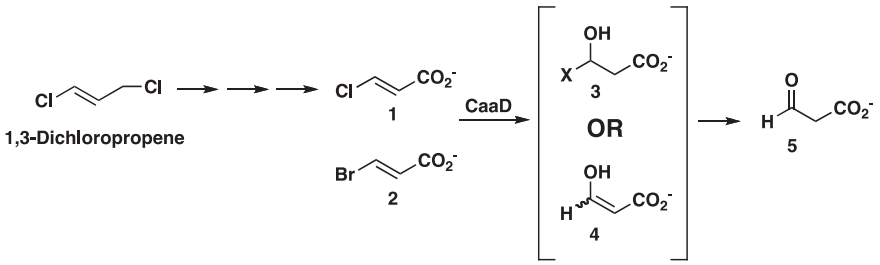
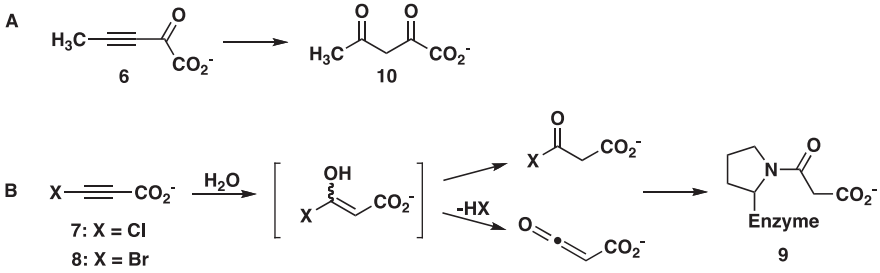


Scheme 1.



Scheme 2.

no detectable activity for the αE52Q mutant and greatly reduced activity for the βP1A mutant [1,9]. The two arginines (αArg-8 and αArg-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 βAsn-39, αPhe-39, and αPhe-50 to the activities of CaaD were assessed by mutagenesis and docking studies. Accordingly, βAsn-39 was replaced with an alanine, αPhe-39 was replaced with an alanine or a threonine, and αPhe-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 αF50A-CaaD-catalyzed reaction (using **2**), were determined and compared to those of the wild-type. In addition, the roles of αArg-8 and αArg-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 αArg-8 and αArg-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 βAsn-39 and the carboxylate group of αGlu-52 is critical for positioning the αGlu-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 αPhe-50 plays a much more significant role in the CaaD reactions than αPhe-39: αPhe-50 is likely critical for maintaining the active site structural integrity and environment.

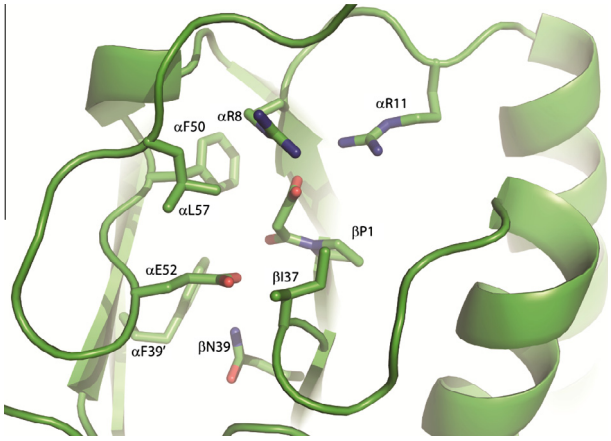


Fig. 1. A close-up of one CaaD active site where the prolyl nitrogen is covalently attached to the 3-oxopropanoate moiety (PDB entry 1S0Y). The residues comprising the active site include βP1, βI37, βN39, αE52, αF39′, αL57, αF50, αR8, and αR11 (clockwise from βP1). The prime indicates that αF39′ is from an adjacent subunit. The core set of residues consists of βP1, αE52, αR8, and αR11. The residues under investigation nearby the core set are βN39, αF39′, and αF50. A putative halide binding pocket consists of βI37, αL57, αF39′, and αF50. The Figure was created with PyMol [20].

2. Materials 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 αR8A, αR11A, αR11K, and αF39A 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