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Resolution of the uncertainty in the kinetic mechanism for the *trans*-3-Chloroacrylic acid dehalogenase-catalyzed reaction

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ABSTRACT

trans- and *cis*-3-Chloroacrylic acid dehalogenase (CaaD and *cis*-CaaD, respectively) catalyze the hydrolytic dehalogenation of their respective isomers and represent key steps in the bacterial conversion of 1,3-dichloropropene to acetaldehyde. In prior work, a kinetic mechanism for the CaaD-catalyzed reaction could not be unequivocally determined because (1) the order of product release could not be determined and (2) the fluorescence factor for the enzyme species, *E*PQ* (where P = bromide and Q = malonate semialdehyde, the two products of the reaction) could not be assigned. The ambiguities in the model have now been resolved by stopped-flow experiments following the reaction using an active site fluorescent probe, αY60W-CaaD and 3-bromopropiolate, previously shown to be a mechanism-based inhibitor of CaaD, coupled with the rate of bromide release in the course of CaaD inactivation. A global fit of the combined datasets provides a complete minimal model for the reaction of αY60W-CaaD and 3-bromoacrylate. In addition, the global fit produces kinetic constants for CaaD inactivation by 3-bromopropiolate and implicates the acyl bromide as the inactivating species. Finally, a comparison of the model with that for *cis*-CaaD shows that for both enzymes turnover is limited by product release and not chemistry.

trans- and *cis*-3-Chloroacrylic acid dehalogenase, designated CaaD and *cis*-CaaD, respectively, catalyze the hydrolytic dehalogenation of the corresponding isomer of 3-chloroacrylic acid (**2** or **3** in Scheme 1) to yield malonate semialdehyde (**4**) and HCl [1–4]. The dehalogenation step is a critical one in bacterial degradative pathways for 1,3-dichloropropene (**1**) [5,6]. The isomeric mixture of **1** represents the active ingredient in commercially available fumigants used to kill nematodes. Subsequent decarboxylation of **4** by malonate semialdehyde decarboxylase (MSAD) produces acetaldehyde (**5**) [7], presumably channeled to the Krebs Cycle, and completes the catabolism of **1** in soil.

The two dehalogenases pose various mechanistic, structural, and evolutionary questions [8]. CaaD is a heterohexamer made up of three heterodimers [2,9]. Each heterodimer consists of an α-subunit (70 amino acids) and a β-subunit (75 amino acids). The accumulated body of evidence suggests a mechanism involving four active site groups, βPro-1, αArg-8, αArg-11, and αGlu-52 [2,3,9,10]. In contrast, *cis*-CaaD is a homotrimer where each monomer consists of 149 amino acids [11]. The enzyme uses two additional groups, His-28 and Tyr-103, along with four active site groups (Pro-1, Arg-70, Arg-73, and Glu-114) that correspond to the ones in CaaD [4,11].

The mechanisms of CaaD and *cis*-CaaD largely parallel one another [8]. In CaaD, αGlu-52 is proposed to activate a water molecule for addition to C-3 of **2**, and the two arginine residues are proposed to bind and polarize the substrate (Scheme 2) [9,10]. Polarization of **2** draws electron density away from C3 to facilitate the addition of water. The combined action produces the enediolate **6** (perhaps not as a distinct entity), which can undergo two fates (routes A and B). In route A, βPro-1 (pK_a ~9.2) provides a proton at C2 to complete the conjugate addition of water and form a chlorohydrin species, **7**. Chemical or enzyme-catalyzed breakdown of **7** expels chloride (as HCl) and affords **4**. In route B, enediolate **6** undergoes an α,β-elimination to produce the enol species **8**. Chemical or enzymatic ketonization of **8** yields **4**. In *cis*-CaaD, Tyr-103 is proposed to assist Glu-114 in the activation of a water molecule and His-28 might function along with Arg-70 and Arg-73 in the binding and polarization of the substrate in the active site [11].

As part of an effort to delineate the similarities and differences

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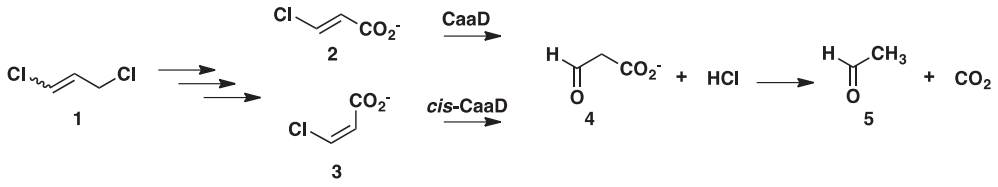
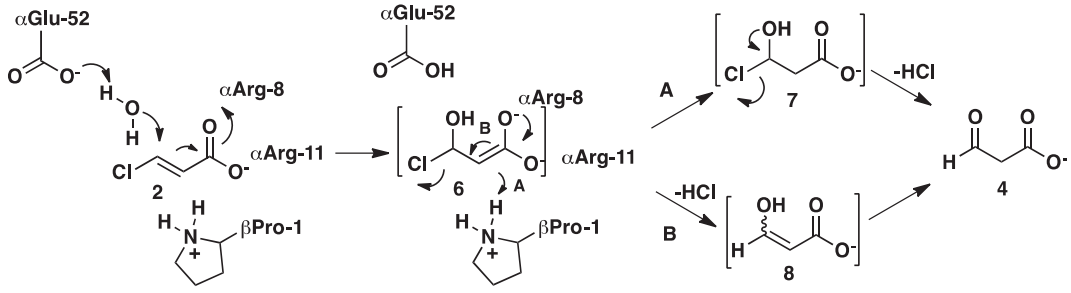


Scheme 1. 1,3-dichloropropene catabolic pathway.



Scheme 2. Catalytic mechanism of CaaD using *trans*-3-chloroacrylic acid (**2**).

between these enzymes, the kinetic mechanism for the CaaD reaction was determined. CaaD lacks a strongly fluorescent amino acid in or near the active site, so one was introduced [12]. The resulting αY60W mutant of CaaD shows little kinetic differences from those of the wild-type, and accurately reports changes in enzyme fluorescence during the CaaD-conversion of *trans*-3-bromoacrylate (**9**, Scheme 3) to **4**, in both the transient state and steady-state realms.

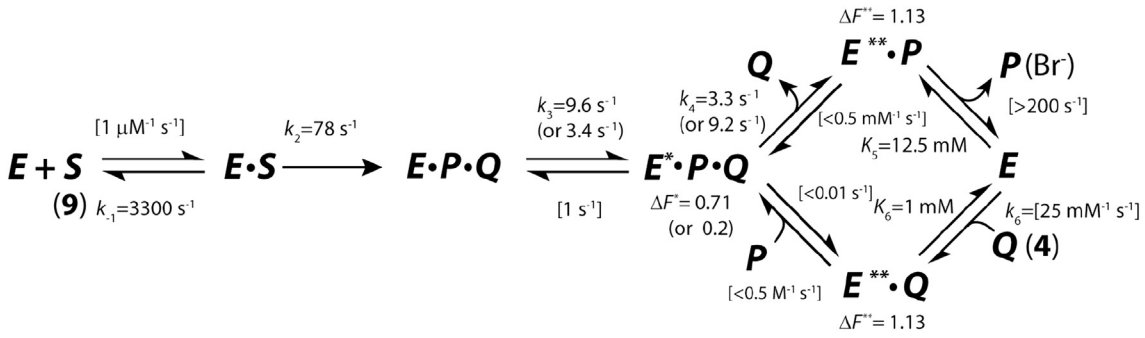
Subsequently, the stopped-flow, rapid chemical quench, ultraviolet spectroscopy, and product (**4** and bromide ion) binding data were subjected to a simultaneous global fit by computational simulation [12]. The fit established a minimal kinetic model for the CaaD reaction with estimates for six individual rate constants (Scheme 4), including chemistry (k_2), product release (k_4) and binding constants for the substrate ($K_D = k_{-1}/k_1$) and products (**4** and bromide, K_5 and K_6, respectively). Limits (lower and upper, respectively) were placed on k_1 and k_{-3}, but their absolute values were not defined by the data. Values in brackets were held fixed during the fitting routines.

However, two features of the model could not be resolved [12].

Scheme 3. Compounds used in the development of the kinetic models.

First, the data set did not define the order of product release (*P*, in Scheme 4) from the E^*PQ species. Binding of *P* to $E^{**}Q$ could only be assessed as weak, with an estimated $K_D > 200$ mM. Hence, it was concluded that product release occurs randomly with a preference for the upper path (in Scheme 4). Second, and more importantly, the data set doesn't explicitly define the fluorescence factor for E^*PQ, which prevents unambiguous resolution of the rate constants for its formation and decay (k_3 and k_4, respectively in Scheme 4), although the data set provides strict upper and lower limits on each parameter. The inability to determine the fluorescence factor appears to be an inherent limitation of the system and is a common problem. Without knowing the absolute concentration of an intermediate one cannot resolve whether the rate constants for the formation and decay follow a fast-slow or slow-fast sequence, as illustrated in Scheme 4 (k_3 and k_4).

In order to determine the fluorescence factor for E^*, and thereby resolve the ambiguity in the rate constants, the reaction of αY60W-CaaD and 3-bromopropiolate (**10**, Scheme 3), a mechanism-based inhibitor of CaaD [3], was followed in stopped-flow experiments. The resulting stopped-flow fluorescence data and the previously determined bromide release data (for **10**) were fit by conventional methods and simulation to yield $k_{inact}/K_{I,app}$, $k_{inact,\ app}$, and an estimate of the rate of bromide release (*P*) from species E^*PR, where *R* is the reactive intermediate that results in CaaD inactivation. The fit also defines unique fluorescent factors for two enzyme species (designated E^* and $E^!$).



Scheme 4. Minimal kinetic mechanism for the reaction of αY60W-CaaD and **9**, where *S* corresponds to **9**, *P* corresponds to bromide, and *Q* corresponds to **4**.