# A persistent pesticide residue and the unusual catalytic proficiency of a dehalogenating enzyme

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Contributed by Richard V. Wolfenden, September 20, 2005

**The soil of potato fields in The Netherlands harbors bacteria with the ability to metabolize 3-chloroacrylic acid, generated by the degradation of a pesticide (1,3-dichloropropene) that entered the environment in 1946. From examination of rate constants at elevated temperatures, we infer that the half-time at 25°C for spontaneous hydrolytic dechlorination of *trans*-3-chloroacrylic acid is 10,000 years, several orders of magnitude longer than half-times for spontaneous decomposition of other environmental pollutants such as 1,2-dichloroethane (72 years), paraoxon (13 months), atrazine (5 months), and aziridine (52 h). With thermodynamic parameters for activation similar to those for the spontaneous hydration of fumarate at pH 6.8, this slow reaction proceeds at a constant rate through the pH range between 2 and 12. However, at the active site of the enzyme 3-chloroacrylate dehalogenase (CaaD), isolated from a pseudomonad growing in these soils, hydrolytic dechlorination proceeds with a half-time of 0.18 s. Neither $k_{cat}$ nor $k_{cat}/K_m$ is reduced by increasing solvent viscosity with trehalose, implying that the rate of enzymatic dechlorination is controlled by chemical events in catalysis rather than by diffusion-limited substrate binding or product release. CaaD achieves an ≈$10^{12}$-fold rate enhancement, matching or surpassing the rate enhancements produced by many enzymes that act on more conventional biological substrates. One of those enzymes is 4-oxalocrotonate tautomerase, with which CaaD seems to share a common evolutionary origin.**

3-chloroacrylate | 3-chloropropene | evolution | *trans*-3-chloracrylate dehalogenase

The active ingredient (1,3-dichloropropene) of the nematocides Shell D-D and Telone II that were first tested on potato fields in 1946 (1) is degraded rapidly to 3-chloroacrylic acid, an unnatural substance with an inherent stability that has not been established. In the presence of pseudomonads cultured from these soils, 3-chloroacrylic acid undergoes hydrolytic dechlorination to malonic semialdehyde and HCl (Fig. 1), and these bacteria can use 3-chloroacrylic acid as their sole carbon and energy source (2). A hydrolytic dehalogenase termed CaaD (*trans*-3-chloroacrylate dehalogenase) is expressed constitutively in *Pseudomonas pavonaceae*. CaaD has been shown to resemble 4-oxalocrotonate tautomerase (4-OT) in its overall structure; both enzymes are equipped with an N-terminal proline residue with a free amino group that seems to play a direct role in catalysis, and the activity of both enzymes depends on the presence of an arginine residue at position 11. Accordingly, these enzymes are considered to share a common evolutionary origin (3–5). In the present work, we sought to determine the rate enhancement produced by CaaD and the thermodynamic basis of that rate enhancement for comparison with the rate enhancements produced by conventional enzymes (including 4-OT) acting on their natural substrates.

## Materials and Methods

To determine rate constants for the uncatalyzed hydrolysis of *trans*-3-chloroacrylate ($k_{non}$), aliquots of this substrate ($8 \times 10^{-3}$ M) in potassium phosphate buffer (0.1 M, pH 6.8) were sealed under vacuum in quartz tubes (4 mm o.d., 0.5 mm wall; obtained

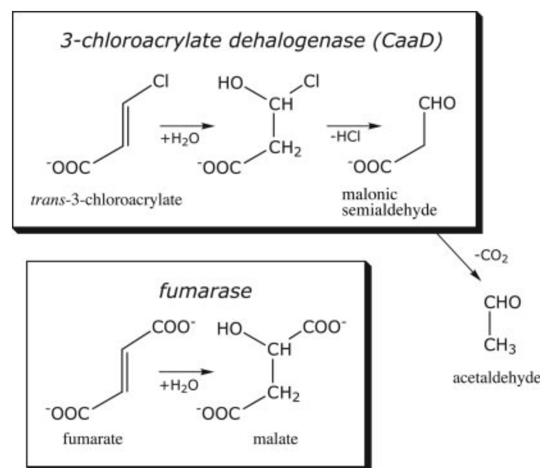


**Fig. 1.** Reaction catalyzed by CaaD and fumarase.

from GM Associates, Oakland, CA) and incubated for various time intervals at various temperatures (140–240°C). After cooling on ice, samples were diluted in $^2H_2O$ to which pyrazine ($10^{-3}$ M) had been added as a proton-integration standard. Proton NMR was used to verify that the products of the uncatalyzed reaction (acetaldehyde and acetaldehyde hydrate) were the same as those generated by the action of CaaD and to follow the course of hydrolytic dechlorination. Rate constants were calculated from the integrated intensities of reactants and products. Enzyme kinetic assays on CaaD from *P. pavonaceae* (kindly provided by Christian Whitman, University of Texas, Austin) were performed by using a Hewlett–Packard 8452A diode array spectrophotometer after the disappearance of *trans*-3-chloroacrylate ($\Delta\varepsilon_{224\ nm} = -4{,}900\ cm^{-1}{\cdot}M^{-1}$) (6). To determine the influence of temperature on $k_{cat}$ and $K_m$, solutions containing substrate at concentrations ranging from $K_m/3$ to $7 \times K_m$ in potassium phosphate buffer (0.1 M, pH 8.0) were incubated with CaaD (6.1 $\mu$g/ml) at temperatures ranging from 10 to 35°C. To investigate the effects of viscosity on $k_{cat}$ and $k_{cat}/K_m$, trehalose was used to produce relative viscosities ranging from 1 to 6 as determined with a Cannon–Fenske viscometer at 25°C as described previously (7).

## Results and Discussion

At 150°C in potassium phosphate buffer, *trans*-3-chloroacrylate was converted spontaneously to an equilibrium mixture of acetaldehyde and its covalent hydrate as a simple first-order process, as indicated by proton NMR spectroscopy. Rate constants for the uncatalyzed reaction did not vary significantly in

Conflict of interest statement: No conflicts declared.

Abbreviations: CaaD, *trans*-3-chloroacrylate dehalogenase; 4-OT, 4-oxalocrotonate tautomerase.

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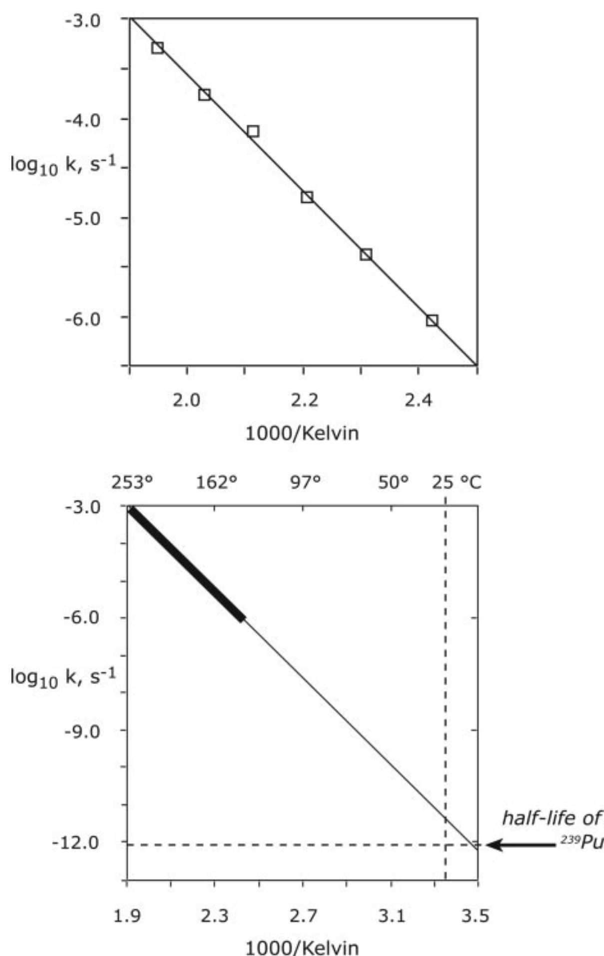


**Fig. 2.** Effect of temperature on the first-order rate constant for dehalogenation of *trans*-3-chloroacrylic acid (0.01 M) in potassium phosphate buffer (0.1 M, pH 8.0).

potassium formate, acetate, or phosphate and carbonate buffers (0.1 M) distributed over the pH range between 1 and 12 or with changing potassium phosphate buffer concentrations (ranging from $10^{-4}$ to 0.1 M) at 150°C and pH 6.8. An Arrhenius plot of rate constants observed for the uncatalyzed reaction at pH 6.8, as a function of temperature, was linear (Fig. 2) and could be extrapolated to yield $k_{non} = 2.2 \times 10^{-12}$ s$^{-1}$ at 25°C.

Of the unnatural substances that have been introduced into the environment as a result of human activity, 3-chloroacrylate is unusual in its resistance to spontaneous hydrolysis (Fig. 3). Indeed, the temperature dependence of chloroacrylate hydrolysis is such that its $t_{1/2}$ becomes identical at 19°C to the temperature-independent $t_{1/2}$ for $^{239}$Pu decay (24,000 years) (Fig. 2). In view of the rate of application of the precursor 1,3-dichloropropene to potato fields (17 g/m$^2$ per year), that resistance to hydrolysis would be a matter for practical concern but for the benign activities of microorganisms that can use 1,3-dichloropropene, 3-chloroallyl alcohol, or 3-chloroacrylate as their sole sources of carbon and energy (2).

Rate constants and thermodynamic parameters of activation observed for the enzymatic ($ES \rightarrow ES^{\ddagger}$) and nonenzymatic ($S \rightarrow S^{\ddagger}$) dehalogenation of *trans*-3-chloroacrylate are shown in Fig. 4. Comparison of the values for the catalyzed and uncatalyzed reactions indicates that this enzyme enhances the reaction rate by a factor of $2 \times 10^{12}$ ($\Delta\Delta G^{\ddagger} = -16.7$ kcal/mol) entirely by reducing the enthalpy of activation ($\Delta\Delta H^{\ddagger} = -17.3$ kcal/mol), whereas the entropy of activation ($\Delta\Delta S^{\ddagger} = -1.2$ kcal/mol) is

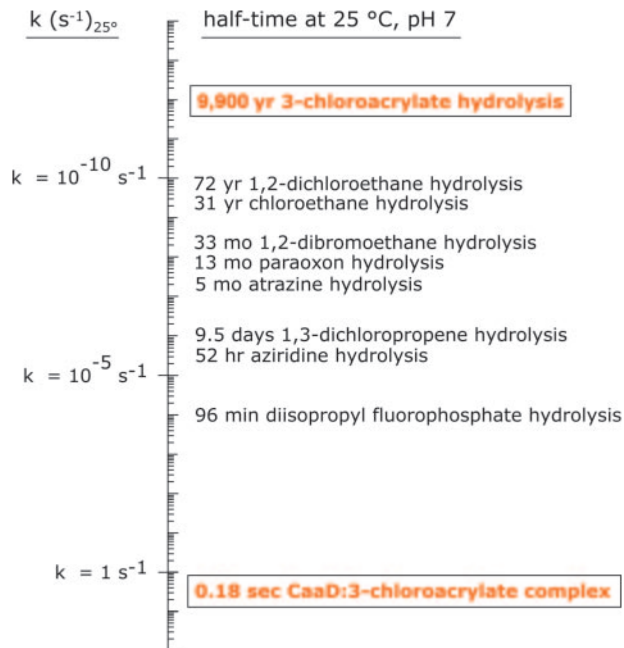


**Fig. 3.** Rate constants for the uncatalyzed decomposition at 25°C of 3-chloroacrylate (this work), 1,2-dichloroethane (18), chloroethane (19), 1,2-dibromoethane (19), paraoxon (20), atrazine (21), 1,3-dichloropropene (22), and aziridine (23).

slightly less favorable on the enzyme than in solution. The entirely enthalpic basis of this rate enhancement would seem understandable if polar forces of attraction (rather than substrate approximation or hydrophobic effects) were responsible for binding the altered substrate in the transition state. The active site of CaaD is lined with charged residues that cannot be altered without inactivating the enzyme and are believed to stabilize the transition state in this way (3, 4, 8).

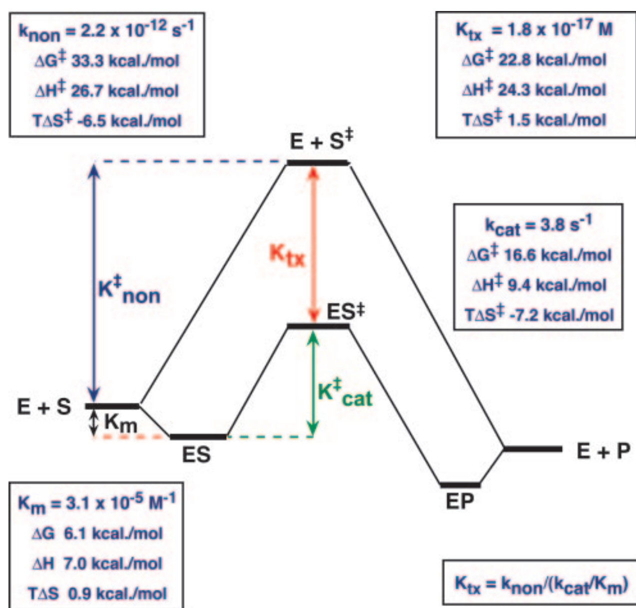


**Fig. 4.** Rate constants, equilibrium constants, and thermodynamics of activation for the CaaD-catalyzed and uncatalyzed dehalogenation of *trans*-3-chloroacrylic acid at 25°C, determined in potassium phosphate buffer (0.02 M, pH 8.0).

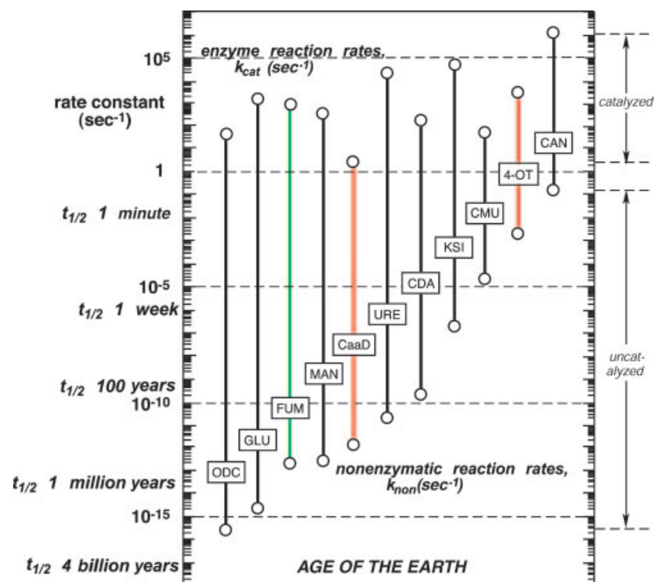


**Fig. 5.** Rate constants for some enzyme-catalyzed and uncatalyzed reactions at 25°C, including orotidylate decarboxylase (ODC), β-amylase (GLU), fumarase (FUM) (13), mandelate racemase (MAN), CaaD (this work), urease (URE), cytidine deaminase (CDA), ketosteroid isomerase (KSI), chorismate mutase (CMU), 4-OT (18), and carbonic anhydrase (CAN). For references, see Table 1.

Compared with the values observed for more familiar enzymes that act along the central pathways of metabolism, the values of $k_{cat}$ (3.8 $s^{-1}$) and $k_{cat}/K_m$ ($1.2 \times 10^5$ $s^{-1} \cdot M^{-1}$) for CaaD are not remarkable for their absolute magnitude (for a recent table of examples, see ref. 9). However, when the difficulty of the uncatalyzed reaction is taken into consideration, it becomes apparent that CaaD, despite the artificial nature of its substrate, produces a rate enhancement that equals or surpasses the rate enhancement produced by many enzymes that act on natural substrates (Fig. 5). Thus, the rate enhancement ($2 \times 10^{12}$-fold) produced by CaaD, acting on 3-chloroacrylate, is comparable in magnitude with those produced by cytidine deaminase (7), urease (10), and proteases (11) that catalyze somewhat similar addition–elimination reactions. The rate enhancement produced by CaaD is somewhat exceeded by the $3 \times 10^{15}$-fold rate enhancement produced by fumarase (Fig. 5), an unusually efficient enzyme that catalyzes a water-addition reaction with spontaneous heat and entropy of activation ($\Delta H^{\ddagger}$ = 28.9 kcal/mol; $T\Delta H^{\ddagger}$ = −6.8 kcal/mol) (12) that are similar to those reported here for the hydrolytic dechlorination of 3-chloroacrylate ($\Delta H^{\ddagger}$ = 26.7 kcal/mol; $T\Delta H^{\ddagger}$ = −6.5 kcal/mol). [The possibility that these rates might be similar was predicted by Azurmendi *et al.* (8).]

The effectiveness of CaaD as a catalyst can also be appreciated by considering the apparent dissociation constant ($K_{tx}$) of CaaD from the altered substrate in the transition state, which is obtained by dividing the rate constant for the uncatalyzed reaction ($k_{non}$) by the second-order rate constant for the enzyme-catalyzed reaction ($k_{cat}/K_m$) (13). For CaaD, that value is $1.8 \times 10^{-17}$ M. To appreciate the physical significance of that value [or its reciprocal, the catalytic proficiency (14)], it is helpful to know the physical significance of $k_{cat}$ and $k_{cat}/K_m$ (13). In some reactions, such as that catalyzed by triosephosphate isomerase (15), $k_{cat}/K_m$ represents the second-order rate constant for the enzyme–substrate encounter rather than the second-order rate constant for chemical transformation of the substrate at the active site of the enzyme. Also, in some cases, typified by the reaction catalyzed by alcohol dehydrogenase (16), $k_{cat}$ represents the rate of release of a product rather than the rate of chemical transformation of the enzyme–substrate complex. In either of those cases, $K_{tx}$ represent only an upper limit on the value of the dissociation constant of the enzyme–substrate complex in the transition state (13). In the case of CaaD, we found that $k_{cat}/K_m$

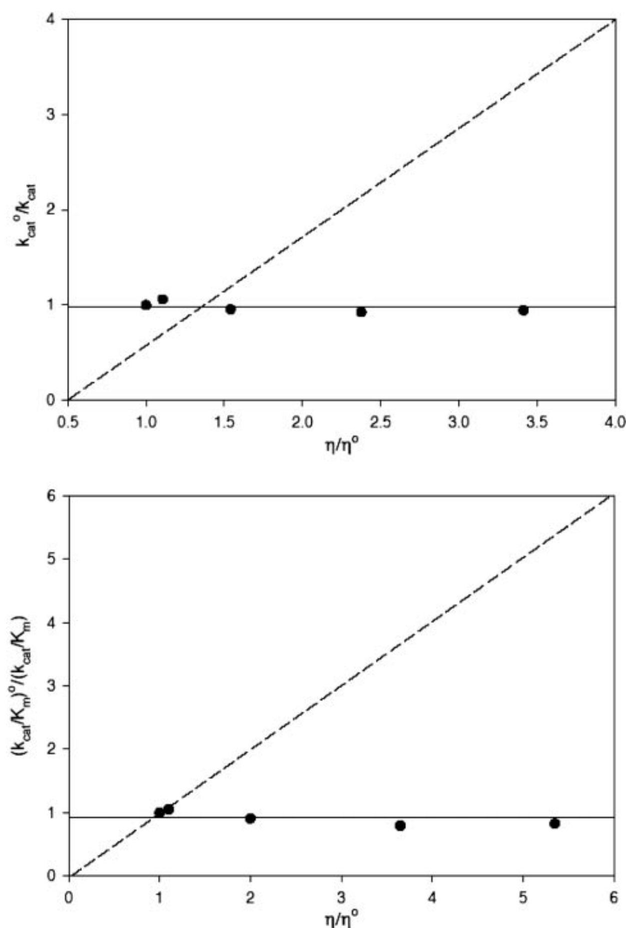


**Fig. 6.** Influence of relative viscosity in the presence of added trehalose on $k_{cat}$ (*A*) and $k_{cat}/K_m$ (*B*) for CaaD in potassium phosphate buffer (0.02 M, pH 8.0) at 25°C.

**Table 1. Rate enhancements and transition state dissociation constants ($K_{tx}$)**

| Enzyme | $k_{cat}/k_{non}$ | $K_{tx}$, M | Source |
|---|---|---|---|
| Fructose 1,6-bisphosphatase | $1.1 \times 10^{21}$ | $1.3 \times 10^{-27}$ | 25 |
| Orotidylate decarboxylase | $1.4 \times 10^{17}$ | $4.4 \times 10^{-24}$ | 14 |
| Arginine decarboxylase | $7 \times 10^{19}$ | $9 \times 10^{-24}$ | 26, 27 |
| β-Amylase | $7 \times 10^{17}$ | $1.0 \times 10^{-22}$ | 28 |
| Fumarase | $3.5 \times 10^{15}$ | $1.0 \times 10^{-21}$ | 12 |
| Staphylococcal nuclease | $5.6 \times 10^{14}$ | $1.7 \times 10^{-20}$ | 14 |
| Urease | $3 \times 10^{15}$ | $1.2 \times 10^{-18}$ | 10 |
| Mandelate racemase | $1.7 \times 10^{15}$ | $2 \times 10^{-19}$ | 29 |
| Carboxypeptidase b | $1.3 \times 10^{13}$ | $3 \times 10^{-18}$ | 11 |
| CaaD | $1.8 \times 10^{12}$ | $1.8 \times 10^{-17}$ | This work |
| Cytidine deaminase | $1.1 \times 10^{12}$ | $1.0 \times 10^{-16}$ | 7 |
| Phosphotriesterase | $2.8 \times 10^{11}$ | $2 \times 10^{-16}$ | 30 |
| Ketosteroid isomerase | $4 \times 10^{11}$ | $6 \times 10^{-16}$ | 14 |
| Triosephosphate isomerase | $1.0 \times 10^{9}$ | $1.8 \times 10^{-14}$ | 14 |
| 4-OT | $2 \times 10^{7}$ | $9 \times 10^{-12}$ | 17 |
| Chorismate mutase | $1.9 \times 10^{6}$ | $2.4 \times 10^{-11}$ | 14 |
| Carbonic anhydrase | $7.7 \times 10^{6}$ | $1.1 \times 10^{-9}$ | 14 |
| Cyclophilin | $4.6 \times 10^{5}$ | $1.9 \times 10^{-9}$ | 14 |

did not decrease with increasing solvent viscosity in the presence of added trehalose (Fig. 6*B*). Accordingly, $k_{cat}/K_m$ seems to be limited not by diffusion but by chemical events in substrate transformation. $k_{cat}$ also is not reduced by increasing solvent viscosity (Fig. 6*A*). Accordingly, $k_{cat}$ seems to describe the chemical transformation of the substrate at the active site of the enzyme. These findings are consistent with the view that $K_{tx}$ represents the actual dissociation constant of the enzyme–substrate complex in the transition state. The $K_{tx}$ value observed here for CaaD, $1.8 \times 10^{-17}$ M, is lower (and its catalytic proficiency higher) than values that have been reported for some enzymes that catalyze more conventional reactions (Table 1).

As far as is known, 3-chloroacrylic acid first appeared in the environment in 1946. Comparisons of structure and mechanism have furnished persuasive evidence that CaaD may have evolved from 4-OT or from a common progenitor (3–5). In view of its possibly recent origin, the proficiency of CaaD as a catalyst seems surprising. Moreover, Fig. 3 shows that CaaD produces a rate enhancement ($2 \times 10^{12}$-fold) that greatly exceeds the rate enhancement ($2 \times 10^{7}$-fold) produced by the action of 4-OT on its natural substrate, 2-hydroxymuconate (17). These are not the properties expected of a newly evolved enzyme. Because CaaD lacks any apparent leaving-group site for chloride, and for other reasons, it has been suggested that the apparently novel catalytic activity of CaaD may be a fortuitous side reaction of an enzyme that evolved to act on a natural substrate that remains to be identified (8). That possibility would be strengthened if the gene for CaaD were found to exist in pseudomonads from fields that had never been treated with pesticide.

We thank Christian P. Whitman for acquainting us with this problem and providing a gift of recombinant CaaD. This work was supported by National Institutes of Health Grant GM-18325.


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