Insights into the catalytic mechanism of a bacterial hydrolytic dehalogenase that degrades the fungicide chlorothalonil

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Chlorothalonil (2,4,5,6-tetrachloroisophtalonitrile; TPN) is one of the most commonly used fungicides in the United States. Given TPN’s widespread use, general toxicity, and potential carcinogenicity, its biodegradation has garnered significant attention. Here, we developed a direct spectrophotometric assay for the Zn(II)-dependent, chlorothalonil-hydrolyzing dehalogenase from Pseudomonas sp. CTN-3 (Chd), enabling determination of its metal-binding properties; pH dependence of the kinetic parameters $k_{cat}$, $K_m$, and $k_{cat}/K_m$; and solvent isotope effects. We found that a single Zn(II) ion binds a Chd monomer with a $K_d$ of 0.17 μM, consistent with inductively coupled plasma MS data for the as-isolated Chd dimer. We observed that Chd was maximally active toward chlorothalonil in the pH range 7.0–9.0, and fits of these data yielded a $pK_{ES1}$ of 5.4 ± 0.2, a $pK_{ES2}$ of 9.9 ± 0.1 ($k'_{cat} = 24 ± 2 \text{ s}^{-1}$), a $pK_E1$ of 5.4 ± 0.3, and a $pK_E2$ of 9.5 ± 0.1 ($k'_{cat}/K_m = 220 ± 10 \text{ s}^{-1} \text{ mM}^{-1}$). Proton inventory studies indicated that one proton is transferred in the rate-limiting step of the reaction at pH 7.0. Fits of UV-visible stopped-flow data suggested a three-step model and provided apparent rate constants for intermediate formation (i.e. a $K_2$ of 35.2 ± 0.1 s$^{-1}$) and product release (i.e. a $K_3$ of 1.1 ± 0.2 s$^{-1}$), indicating that product release is the slow step in catalysis. On the basis of these results, along with those previously reported, we propose a mechanism for Chd catalysis.

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The gene from Pseudomonas sp. CTN-3 that encodes for Chd was synthesized with optimized Escherichia coli codon usage and includes a TEV protease cleavage site followed by a emerging as a major environmental issue (6, 7). TPN is also a human skin and eye irritant that can cause severe gastrointestinal issues. Animal studies involving mice have shown that TPN can cause kidney cancer, so it has been classified by the United States Environmental Protection Agency as a probable human carcinogen (3). Given the widespread use of TPN and its toxicity, its biodegradation and environmental clean-up has become a topic of significant importance (8).

Characterized pathways for biological dehalogenation of organics include oxidative, reductive, and thiolytic mechanisms (9–15). However, selective partial dehalogenation of TPN can also be catalyzed by a hydrolytic process that converts TPN to 4-hydroxytrichloroisophtalonitrile (4-OH-TPN) and chloride (Scheme 1) (7, 16, 17). Several bacterial strains harbor a gene that has been shown to be responsible for TPN dehalogenation (18–20). Each of these gene products exhibits remarkable (>95%) identity and requires Zn(II) as a cofactor for catalysis (Fig. 1).

The best-characterized enzyme within this group is the TPN dehalogenase from Pseudomonas sp. CTN-3 (Chd, EC 3.8.1.2) (7, 21). Chd contains a conserved Zn(II)-binding domain similar to enzymes in the metallo-β-lactamase superfamily and was proposed to be monomeric in solution (7). At least two His residues (His-128 and His-157) along with three Asp (Asp-45, Asp-130, and Asp-184), a Ser (Ser-126), and a Trp (Trp-241) were reported to be catalytically essential based on site-directed mutagenesis studies. In addition, it was reported that the Zn(II) ions associated with Chd could be substituted with Cd(II), Co(II), Ca(II), or Mn(II) and provide active or even hyperactive enzymes (21). Whereas the initial biological characterization of Chd has provided some insight into how molecular structure controls enzyme function, the mechanism of action remains entirely unknown.

Herein, we report a new continuous spectrophotometric assay for Chd that has allowed the detection of a Chd reaction intermediate using UV-visible stopped-flow spectroscopy with TPN as the substrate. From these stopped-flow data, along with metal-binding and kinetic studies including pH and solvent isotope effect studies, we propose the first catalytic mechanism for Chd.

Results

Protein expression and purification

The gene from Pseudomonas sp. CTN-3 that encodes for Chd was synthesized with optimized Escherichia coli codon usage and includes a TEV protease cleavage site followed by a...
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polyhistidine (His6) affinity tag engineered onto the C terminus. Expression of Chd and purification using immobilized metal affinity chromatography (IMAC) resulted in ~12 mg/liter soluble Chd enzyme. SDS-PAGE reveals a single polypeptide band at ~36 kDa (Fig. S1), consistent with previous studies (7, 21). However, size-exclusion chromatography indicates that Chd exists primarily as a dimer (~72 kDa) in solution in 50 mM HEPES buffer, pH 7.0, at 25 °C.

Spectrophotometric enzymatic assay

A continuous spectrophotometric enzymatic assay for Chd was developed by directly detecting 4-OH-chlorothalonil, the product of TPN hydrolysis by Chd, at 345 nm (ε345 = 3.5 mM⁻¹ cm⁻¹). This region contains no detectable substrate absorption (5). All kinetic data were recorded on a temperature-controlled Shimadzu UV-2450 spectrophotometer in 50 mM HEPES buffer, pH 7.0, at 25 °C, over a 60-s time period. Plots of the initial rate of hydrolysis of various concentrations of TPN were fit to the Michaelis–Menten equation, which provides a kinetic model. These data are consistent with inductively coupled atomic emission spectroscopy (ICP-AES) data obtained on purified Chd, which revealed that ~0.9 eq of zinc bind tightly to Chd per monomer. No other first row transition metal ions were detected via ICP-MS (<10 ppb).

pH dependence of the kinetic parameters

The kinetic parameters $K_{cat}$, $k_{cat}$, and $k_{cat}/K_m$ were determined as a function of pH using TPN as the substrate. Chd was found to exhibit a bell-shaped curve for plots of activity versus pH over the pH range 4–10. The maximum catalytic activity occurred in the range of pH 6.5–9.2. Log($k_{cat}$) and log($k_{cat}/K_m$) were fit to Equations 4 and 5 (25), respectively,

\[
\log k_{cat} = \log \left( \frac{k_{cat}}{1 + 10^{pK_{a1} - pH} + 10^{pK_{a2} - pH}} \right) \quad \text{(Eq. 4)}
\]

\[
\log k_{cat}/K_m = \log \left( \frac{k_{cat}}{1 + 10^{pK_{a1} - pH} + 10^{pK_{a2} - pH}} \right) \quad \text{(Eq. 5)}
\]

where $k_{cat}$ is the theoretical maximal velocity; $k_{cat}/K_m$ is the theoretical maximal catalytic efficiency; $K_{ES1}$ is the ionization constant of the ES complex, which affects the acidic side of the pH curve while $K_{ES2}$ reflects the basic side; and $K_{E1}$ and $K_{E2}$ are ionization constants for an acidic and basic group, respectively, on the free enzyme or free substrate. Inspection of a plot of log($K_m$) versus pH (Fig. 3) reveals that $K_m$ exhibits a broad minimum over pH 5.5–7.5. A plot of log($k_{cat}$) versus pH provided a bell-shaped curve that was fit to Equation 4 (Fig. 3), providing a $pK_{ES1}$ value of 5.4 ± 0.2, a $pK_{ES2}$ value of 9.9 ± 0.1, and a $k_{cat}$ value of 24 ± 2 s⁻¹. Similarly, plots of log($k_{cat}/K_m$) versus pH were fit to Equation 5, providing a $pK_{E1}$ value of 5.4 ± 0.3, a $pK_{E2}$ value of 9.5 ± 0.1, and a $k_{cat}/K_m$ value of 220 ± 10 s⁻¹ mm⁻¹ (Fig. 3).

Solvent isotope effect studies

$k_{cat}$ for TPN was measured at several ratios of $D_2O/\text{H}_2O$ and the results are plotted in Fig. 4 as atom fraction of deuterium versus $V_n/V_0$, where $V_n$ is the observed velocity at $n$ fraction of deuterium and $V_0$ is the observed velocity in 100% $\text{H}_2\text{O}$. Proton inventories and fractionation factors were obtained by fitting the experimental values for $V_n/V_0$ to equations derived from the Gross–Butler equation (Equation 6) (26),

\[
V_n/V_0 = \frac{\prod_{i=0}^{n} (1 - n + n\phi_i^I)}{\prod_{i=0}^{n} (1 - n + n\phi_i^P)} \quad \text{(Eq. 6)}
\]

where $V_n$ is the number of protons transferred in the transition state, whereas $V_0$ is the number of protons transferred in the reactant state, and $\Phi$ is the fractionation factor. Fitting revealed a linear relationship ($\phi_1 = 1, \phi_0 = 0$), indicating that one proton is transferred in the transition state when $\Phi = 1$ (Equation 7) (27),

\[
V_n = V_0 \times (1 - n + n\phi_1 \times n) \quad \text{(Eq. 7)}
\]

where the experimental $\phi_1$ value is 0.17, whereas the theoretical value of $\phi_1$ is 0.18 ($R^2 = 0.99$) (Fig. 4).
Rhodococcus sp. XF-6
Rhodococcus sp. XF-3
Pseudomonas sp. CTN-3
Rhodococcus sp. XF-8
Rhizobium sp. CTN-15
Ochrobactrum lupini
Bordetella sp. CTN-10
Ochrobactrum sp. CTN-11
Caulobacter sp. CTN-14

** Figure 1. Chd sequence alignments for nine bacterial species. Yellow, proposed active-site motif. **
Calculation of the partial solvent isotope effect provides an alternative way to determine the number of protons transferred in the transition state (28). At \( n = 0.5 \), the theoretical solvent isotope effect for a process involving \( N \) protons can be estimated using Equation 8, a generalization of Equations 6 and 7 (29),

\[
V_{0.5}/V_1 = [(1 - n_{0.5})(V_{0.5}/V_1)^{1/N} + n_{0.5}]^N \quad (\text{Eq. 8})
\]

where \( V_1 \), \( V_{0.5} \), and \( V_{0.50} \) are the specific activities at 100% D2O, 0% D2O, and 50% D2O, respectively. \( V_{0.5}/V_1 \) represents the midpoint partial solvent isotope effect at 50% D2O, whereas \( V_{0.5}/V_1 \) is the total isotope effect ((velocity in 100% 1H2O)/(velocity in 100% D2O)). The experimental midpoint partial isotope effect was 2.98, and the calculated midpoint partial isotope effect for a one-proton transfer (Equation 8) was found to be 2.99 (Table 1). For comparison purposes, the midpoint partial isotope effect calculated for a two-proton transfer in the transition state is 4.03.

**Stopped-flow experiments**

Steady-state kinetic data were obtained using a 0.25 mM buffered solution of TPN at pH 7.0 and 4 °C. Because of the observed decrease in \( k_{\text{cat}} \) at lower pH values, stopped-flow spectrophotometric data were also collected at 4 °C and pH 5.0; however, the reaction still remained too fast to obtain pre-steady-state kinetic data. However, when stopped-flow experiments were performed in 99% D2O in 50 mM acetate buffer at pH 5.0 and 4 °C with 250 \( \mu \)M TPN, a burst of absorbance was observed (Fig. 5) that could be modeled using a two-component expression containing linear and exponential terms (Equation 9) (30, 31),

\[
[A] = A_0(1 - e^{-k_{\text{cat}t}}) + k_{\text{cat}t} \quad (\text{Eq. 9})
\]

where \([P]\) is the product concentration, \([E]\) is the enzyme concentration, \(A_0\) is the burst amplitude, \(k_{\text{obs}}\) is the overall rate constant, and \(k_{\text{cat}}\) is the turnover number (\(R^2 = 0.98\)). As this experiment is performed under saturating substrate concentrations, \(k_1'\) for formation of the Michaelis complex is large. Therefore, it does not influence the multiple-turnover kinetics, and theoretical modeling of the data returns information only on the formation of a post-Michaelis intermediate \(k_2'\) and product release \(k_3'\) (Equations 10 and 11) (Table 2) (30).

\[
A_0 = \left( \frac{k'}{k_2' + k_1'} \right)^2 \quad (\text{Eq. 10})
\]

\[
k_{\text{obs}} = k_2' + k_3' \quad (\text{Eq. 11})
\]

Fits of these data (Fig. 5) provided apparent rate constants \(k_2'\) of 35.2 ± 0.1 s\(^{-1}\) and \(k_3'\) of 1.1 ± 0.2 s\(^{-1}\). The \(k_{\text{cat}}\) of the overall...
Figure 4. Plot of \( V_{\text{cat}}/V_{0} \) versus atom fraction of deuterium for Chd at pH 7.0. Each data point was obtained in triplicate in 50 mM HEPES buffer under various ratios of D\(_2\)O/H\(_2\)O at 25 °C and 0.25 mM TPN. Solid line, data fit by a linear equation; dashed line, direct fit to Equation 4 with fractionation \( \Phi_{\text{r}} = 1 \), \( \Phi_{\text{i}} = 0.18 \). Error bars, S.D.

reaction was calculated to be \( 1.1 \pm 0.1 \text{ s}^{-1} \), in good agreement with the value calculated using Equation 12, which provided a \( k_{\text{cat}} \) value of \( 1.08 \pm 0.02 \text{ s}^{-1} \).

\[
k_{\text{cat}} = \frac{k'_{\text{cat}}}{k_{3} + k'_{3}}
\]  
(Eq. 12)

These data were compared with experimentally determined steady-state kinetic data obtained at 4 °C for Chd using 250 \( \mu \text{M} \) TPN as the substrate at pH 5.0 in 99% D\(_2\)O acetate buffer. Under these conditions, \( k_{\text{cat}} = 1.08 \pm 0.01 \text{ s}^{-1} \) and \( K_{m} = 71 \pm 3 \text{ \mu M} \) (Table 2).

Discussion

The prevailing dogma is that biological dechlorination reactions are catalyzed by oxidative, reductive, or thiolytic dehalogenation processes (9–15). A relatively unknown biological dehalogenation process involves hydrolysis of a C–Cl bond (32). Chd, a Zn(II)-dependent enzyme, has been shown to catalyze the hydrolytic dehalogenation of TPN to 4-OH-TPN and chloride (Scheme 1) under ambient conditions (7, 21). As Chd can hydrolyze an aromatic C–Cl bond, understanding the inorganic and biological chemistry of Chd will provide insight into its catalytic mechanism, which in turn will assist in the development of biocatalysts or small biomimetic catalysts that can be used in the environmental clean-up of TPN. To date, no catalytic mechanism has been proposed for Chd, in part because of the lack of an enzymatic assay that allows for the direct detection of product, which has prevented detailed kinetic studies.

To overcome this obstacle, a spectrophotometric kinetic assay was developed that directly detects the formation of 4-OH-chlorothalonil at 345 nm, a wavelength where there is little or no TPN absorbance (5). Initial control reactions were performed with saturating amounts of TPN (250 \( \mu \text{M} \)) in 50 mM HEPES buffer at pH 7 and 25 °C in the absence of Chd by monitoring absorptions between 300 and 400 nm to determine whether any TPN hydrolysis occurred under the experimental conditions used. The addition of 1 \( \mu \text{M} \) Zn(II) to these reaction mixtures also produced no detectable absorption at 345 nm.

With no increase in absorbance observed, Chd was added to a final concentration of 10 \( \mu \text{M} \), resulting in a steady increase in absorption at 345 nm. The rate of increase at 345 nm was highly reproducible and dependent on the concentration of Chd and TPN as well as the temperature and pH of the reaction mixture. At temperatures above 30 °C, gradual inactivation occurs, which is indicative of Chd denaturation. Having established the viability of directly detecting the product of TPN hydrolysis, the kinetic parameters \( k_{\text{cat}} \) and \( K_{m} \) were determined at pH 7.0 and 25 °C over a 60-s time period. Plots of [TPN] versus initial rate were fit to the Michaelis–Menten equation, which provided a \( k_{\text{cat}} \) value of \( 24 \pm 2 \text{ s}^{-1} \) and a \( K_{m} \) value of \( 110 \pm 30 \text{ \mu M} \), in good agreement with values reported previously using a noncontinuous HPLC-based assay performed under similar reaction conditions (7, 21).

The development of a continuous spectrophotometric assay for Chd has allowed us to ask and answer several basic biological and mechanistic questions, such as the following. How many active site metal ions are required for full enzymatic activity? How many ionizable groups are required for catalysis? How many protons are transferred in the transition state? What is the rate-limiting step in the reaction? It has been suggested that Chd requires two Zn(II) ions to be fully active and that these Zn(II) ions form a dinuclear active site (7, 21). However, ICP-AES data obtained on as-purified Chd revealed that ~0.9 Zn(II) ions bind per monomer, and size-exclusion chromatography indicates that Chd exists primarily as a dimer (~72 kDa) in solution.

Activity titrations indicated that maximum catalytic activity is observed with only one Zn(II) ion per monomer of Chd with an intrinsic \( K_{d} \) value of 0.17 \( \mu \text{M} \), suggesting that any other Zn(II) binding is unrelated to catalysis. It should be noted that in previous studies, the His\(_{6}\) tag was not removed before kinetic data were obtained (7, 21). As His\(_{6}\) tags have high affinity for Zn(II) ions (33), it is possible that adventitious metal binding to the His\(_{6}\), tag led to the suggestion that more than one metal ion is required for catalysis.

Quantitative analysis of the pH dependence of Chd activity suggested (c.f. Ref. 34) that one catalytically competent ionizable group with \( pK_{\text{ES1}} \approx 5.4 \) must be deprotonated in the ES complex, and another with \( pK_{\text{ES2}} \approx 9.9 \) must be protonated, respectively, to facilitate catalysis. Assignment of the observed \( pK_{\text{ES}} \) values is difficult in the absence of an X-ray crystal structure; however, likely candidates for \( pK_{\text{ES1}} \) are the deprotonation of an active site His residue (35) (whose putative \( pK_{a} \) is 5–7 (36)) or an Asp/Glu residue, whereas \( pK_{\text{ES2}} \) might be due to the deprotonation of the leaving group or an active-site residue such as an Arg or Lys (7). Alternatively, \( pK_{\text{ES2}} \) may be due to the deprotonation of a Ser residue that was shown to be required for catalysis (7) or a metal-bound water molecule, depending on which catalytic mechanism is operable.

Analysis of the pH dependence of \( \log(k_{\text{cat}}/K_{m}) \) (c.f. Ref. 35) provided a \( pK_{\text{E1}} \) value of 5.4 and a \( pK_{\text{E2}} \) value of 9.5 for two enzyme-centered ionizable groups, respectively, that are involved in catalysis. \( pK_{\text{E1}} \) is most likely due to an active-site His or an Asp/Glu residue but could also be the deprotonation of the metal-coordinated water molecule. Moreover, the enzyme-centered \( pK_{\text{E2}} \) value, like \( pK_{\text{ES2}} \), is most likely due to the depro-
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Table 1

| Kinetic parameters from solvent isotope study | Experimental value | One proton (calculated) | Two protons (calculated) | General solvation (calculated) |
|---------------------------------------------|--------------------|------------------------|--------------------------|-------------------------------|
| $V_{0.5}/V_1$ | 2.98               | 2.99                   | 4.03                     | 2.34                          |

Figure 5. Stopped-flow experiment data obtained by 50 mM acetate in 99% D$_2$O at 4 °C, pH 5.0. These data were fit (solid line) as an exponential for the pre-steady-state burst phase and with a linear equation for the steady-state region.

Based on these data, the simplest model was used that describes the observed changes in ionization states of active-site residues with changing pH and the number of protons transferred in the transition state (Scheme 2). This model assumes that (i) the substrate-binding step leading to the formation of an enzyme–substrate complex follows steady-state kinetics (e.g. the enzyme, substrate, and enzyme–substrate complex are at equilibrium) and (ii) $k^{-1}_c$ is larger than $k^+_c$, as neither the substrate nor the product are sticky, and $k^+_c + k^-_c$ contains the rate-limiting step (i.e. C–Cl bond breaking or product release). Because there is no clear absorption change as [ES] and intermediate signal within the burst phase, we assume that $k_2$ and $k_3$ are irreversible (30, 31).

An important question in understanding the hydrolysis of TPN by Chd is identity of the rate-limiting step in the catalytic reaction. Pre-steady-state kinetic data indicated that formation of the Michaelis complex is very fast compared with the hydrolysis and product-release steps, and, therefore, the rate constants for the latter two could be estimated from multiple-turnover stopped-flow spectrophotometry. Based on these data, a minimal three-step kinetic model is proposed that allows for fast reversible substrate binding, the formation of a post-Michaelis pre-transition-state intermediate, and the post-transition-state release of product (Scheme 3).

The electron density distribution of free and Zn(II)-bound TPN calculated using Gaussian 9-win (Scheme 4) and the previously reported kinetic and site-directed mutagenesis data (7, 21) suggests two possible catalytic mechanisms for the hydrolysis of TPN by Chd (Fig. 6). We propose that the initial catalytic step involves the binding of the nitrile nitrogen to the active-site Zn(II) ion, which results in the removal of electron density from the aromatic ring activating the ortho-carbon for nucleophilic attack (Scheme 4B) (39, 40). The significantly enhanced electrophilic character of the ortho-carbon upon nitrile binding to Zn(II) suggests that Zn(II) binding activates the ortho-carbon toward nucleophilic attack and may also help to position the ortho-carbon relative to the nucleophile, thus preorganizing the transition state. Based on our kinetic data, an active-site His residue needs to be deprotonated so that it can accept a single proton from a Zn(II)-bound water molecule providing the catalytic nucleophile, which is preorganized adjacent to the activated ortho-carbon of TPN. Once nucleophilic attack occurs, Cl$^{-}$ and 4-OH-TPN are formed and released from the active site, which is the rate-limiting step in catalysis. Finally, a water molecule binds to the active-site Zn(II), thus reforming the active catalyst.

Although the mechanistic proposal involving nitrile binding to the active site Zn(II) ion is logical and has advantages in that binding activates the ortho-carbon for nucleophilic attack and preorganizes the transition state by positioning the Zn(II)-bound hydroxide near the ortho-carbon, there is no experimental evidence to support TPN binding to the active-site Zn(II) ion at this time. Therefore, an alternative pathway involving sub-
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Table 2

|                          | $k_{cat}/s$ (fitting) | $k_{cat}/s$ | $k_{cat}/s$ (observed) |
|--------------------------|----------------------|-------------|------------------------|
| Value                    | 0.93                 | 3.64        | 1.08                   |
| S.E.                     | 0.01                 | 0.29        | 0.01                   |

**Scheme 2. Proposed kinetic model for Chd.** $E(H_2O)$, enzyme containing water molecule at active site; $E^P$, the enzyme in the protonated state; $E^D$, enzyme in the deprotonated state; $S$, substrate; $E(SH_2O)$, enzyme–substrate complex containing a water molecule at the active site; $E^P$, enzyme–substrate complex in the protonated state; $E^D$, enzyme–substrate complex in the deprotonated state; $I$, an intermediate state with deprotonated water; $P$, product.

**Scheme 3. Proposed pre-steady-state model for the dechlorination of TPN by Chd.**

In conclusion, the development of a continuous spectrophotometric assay that allows for the direct detection of the product 4-OH-TPN has allowed the first mechanistic studies to be carried out on the hydrolytic dehalogenase, Chd. Metal titration data indicate that a single metal ion is required for catalytic activity, so Chd can be classified as a mononuclear hydrolytic Zn(II)-dependent enzyme. Chd is a dimer in solution and exhibits a broad kinetic pH dependence with the $V_{max}$ dependent on two ionizable groups, one with $pK_a \approx 5.4$ and one with a $pK_a \approx 9.9$. Solvent kinetic isotope effect studies indicate that one proton is transferred in the transition state, likely due to the breaking of a water $O$–$H$ bond. Pre-steady-state kinetic studies performed under saturating substrate conditions revealed a burst phase followed by a linear, steady-state phase. Determination of $k_2$ and $k_3$ revealed that the product release step is the slow step in the catalytic cycle. Taken together, these findings, along with density functional calculations on TPN in the absence and presence of Zn(II), have allowed two potential catalytic mechanisms to be proposed. Further studies will be required to distinguish between mechanistic pathways A and B.

**Experimental procedures**

**Materials**

Synthesized genes and primers were purchased from Genscript Inc. All other chemicals were purchased from commercial sources and were of the highest quality available.

**Pseudomonas sp. CTN-3 Chd plasmid construction**

Chd sequences were obtained by BLAST search using UniProt (Uniport ID C9EBRS). Proposed active-site motifs for Chd were identified based on the metallo-$\beta$-lactamase superfamily. The predicted gene was synthesized with optimized E. coli codon usage by Genscript Inc. A polyhistidine (His$_6$) affinity tag was engineered onto the C terminus with a TEV cleavage site. The sequence was confirmed using automated DNA sequencing at Functional Biosciences (Madison, WI).

**Expression and purification of Chd**

The Chd plasmid was transformed into BL21(DE3) competent cells (Stratagene), and a single colony was used to inoculate 50 ml of lysogeny broth-Miller culture containing 50 $\mu$g/ml kanamycin with shaking overnight at 37 °C. This culture was used to inoculate a 1-liter culture, and the cells were grown at 37 °C until the $A_{600 nm}$ reached 0.8–1.0. The culture was cooled on ice, induced with 0.1 mM isopropyl $\beta$-D-1-thiogalactopyranoside supplemented with 0.05 mM ZnCl$_2$, and expressed at 25 °C for 16 h. Cells were harvested by centrifugation at 6370 × g.
and 4 °C for 10 min in a Beckman Coulter Avanti JA-10 rotor. Cell pellets were resuspended in 20 mM Tris-HCl buffer containing 50 mM NaCl and 25 mM imidazole at a ratio of 5 ml/g of cells and then sonicated for 4 min (30 s on, 45 s off) at 21 watts using a Misonix sonicator 3000. The crude extract was obtained after centrifugation in a JA-20 rotor at 31,000 × g and 4 °C for 20 min.

Crude extracts of Chd (100 mg) were loaded onto a 5-ml nickel-nitrotriacetic acid Superflow Cartridge (Qiagen) for IMAC using an ÄKTA FPLC P-960. The column was washed with 50 ml of 20 mM Tris-HCl buffer containing 50 mM NaCl and 25 mM imidazole, followed by 50 ml of 20 mM Tris-HCl buffer containing 50 mM NaCl and 75 mM imidazole. The protein was eluted using a linear imidazole gradient (75–500 mM).

Figure 6. Proposed catalytic mechanisms for Chd. Pathway A, an active-site base acts as a proton acceptor for a Zn(II)-bound water. The chlorine atom at the ortho-carbon position is substituted by nucleophilic attack of OH⁻. Pathway B, TPN is stabilized by a hydrogen-bonding interaction with a protonated active-site residue (R₁).
at a flow rate of 2 ml/min. Active protein fractions were pooled and concentrated using 50 mM Tris buffer containing 1 mM EDTA with an Amicon Ultra-15 10,000 molecular weight cutoff centrifugal filter unit (Millipore), resulting in ~12 mg/liter soluble Chd-His6.

The His6 tag was removed by treating His6-tagged Chd with His6-tagged TEV protease (EC 3.4.22.44) for 16 h at 4 °C in 50 mM Tris, pH 8.0. Cleaved protein was concentrated with a Centricon (15,000 molecular weight cutoff; Amicon) to 3 ml and loaded on IMAC to remove the remaining cleaved His6 tag, uncut protein, and the His6-tagged TEV protease, whereas the flow through containing Chd was collected and washed with 50 mM HEPES buffer containing 10% glycerol at pH 7.0. Purified protein samples were analyzed by SDS-PAGE with a 12.5% polyacrylamide SPRINT NEXT GEL™ (Amresco). Gels were stained with Gel Code Blue (Thermo Fisher Scientific). Protein concentration of crude extracts was determined using a Coomassie (Bradford) Protein Assay Kit (Pierce), and concentration of pure protein was determined by measuring the absorbance at 280 nm with a Shimadzu UV-2450 spectrophotometer equipped with a TCC-240A temperature-controlled cell holder. Theoretical molecular weights and protein extinction coefficients were calculated with the ExPaSy compute pI/Mw tool. The molecular weight for Chd was 36,107 g/mol with an extinction coefficient of 42,525 cm⁻¹ M⁻¹. This molecular weight is in good agreement with SDS-PAGE data.

**Chd spectrophotometric assay**

The enzymatic activity of Chd toward TPN was measured using a Shimadzu UV-2450 spectrophotometer equipped with a TCC-240A temperature-controlled cell holder in 1-ml quartz cuvettes. A 1-ml reaction consisted of 50 mM HEPES buffer, 0.01 μM Chd, pH 7.0, at 25 °C and various concentrations of TPN up to 250 μM. The rate of TPN dehalogenation was determined by continuously monitoring the formation of 4-OH-TPN at 345 nm (Δε345 = 3.5 mm⁻¹ cm⁻¹) (5). Data analysis was performed using OriginPro 9.0 (OriginLab, Northampton, MA). The kinetic constants Vmax and Km were calculated by fitting these data to the Michaelis–Menten equation. Vmax values were converted to kcat using the molar mass of Chd. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the production of 1 μmol of TPN/min at 25 °C.

**Metal analysis**

As purified enzyme samples of Chd were digested with concentrated nitric acid at 70 °C for 10 min and then cooled to room temperature. These samples were diluted to a 5-ml total volume with deionized water to give a final nitric acid concentration of 5% and were filtered using 0.2-μm Supor membrane syringe filters (Pall). A nitric acid blank was also prepared. The samples were analyzed using ICP-AES at the Water Quality Center in the College of Engineering at Marquette University (Milwaukee, WI).

**Apoenzyme preparation and Zn(II) Kd determination**

Apo-Chd was obtained by incubating as-purified enzyme in a 15 mM 1,10-phenanthroline, 40 mM EDTA solution under anaerobic conditions for ~24 h. The metal chelators were removed via a PD-Minitrap G10 desalting column followed by dialysis using a Slide-A-Lyzer dialysis cassette for 16 h with Chelex 100—treated 50 mM HEPES at pH 7.0. Titration of Zn(II) into apo-Chd was performed on a Shimadzu UV-2450 spectrophotometer equipped with a TCC-240A temperature-controlled cell holder in 1-ml quartz cuvettes in 50 mM HEPES buffer, 0.25 μM apo-Chd, pH 7.0, at 25 °C. The rate of hydrolysis of TPN (0.25 mM) was monitored as a function of [Zn(II)].

**pH profiles**

The enzymatic activity of 0.01 μM Chd at pH values between 4.0 and 10.2 was measured using TPN as the substrate. The concentration of each buffer used was 50 mM, and the following buffers were used: borate (pH 5.00–8.50), HEPES (pH 5.00–8.50), Tris-HCl (pH 7.00–8.50), HEPES (pH 6.8–7.2), MOPS (pH 6.50–7.00), MES (pH 5.50–6.50), and acetate (pH 3.23–5.50). The kinetic parameters kcat, Km, and kcat/Km were determined using 8–12 different substrate concentrations ranging from 0.2 to 2.5 times the observed Km value at each pH studied. Kinetic parameters and fits to the kinetic curves were obtained using OriginPro 9.0 (OriginLab).

**Solvent isotope effect**

All buffers were prepared from freshly opened bottles of 99.9% [2H]H2O and CH3OH (Aldrich). The buffers used in the preparation of all deuterated buffers were in the anhydrous form. The pD of each buffer used was adjusted by the addition of NaOD or DCI (both 99%+ deuterium content; Acros Organics, Geel, Belgium) and corrected for deuteration by adding 0.4 to the reading of the pH electrode (28).

**Stopped-flow experiments**

Chd activity toward TPN was examined in triplicate using a single mixing Applied Photophysics SX-20 stopped-flow UV-visible spectrophotometer with a 20-μm cell. Chd activity was monitored at 345 nm by acquiring stopped-flow data from 0.005 to 1 s at 4 °C using 8.1 μM enzyme and 250 μM TPN in deuterated 50 mM acetate buffer, pH 5.0. All data were fit using OriginPro 9.0 (OriginLab).

**TPN electron density calculation**

The structures of TPN were drawn using Gaussian View 5.0.8. The bond lengths and overall structures were optimized by DFT calculations at the ground state (basis set: 3-21G) followed by electron density distribution of free TPN by Gaussian 9-win.

**Author contributions**—X. Y. prepared expression plasmid; carried out protein expression, purification, enzymatic assays, and stopped-flow experiments; prepared samples for metal analysis; and analyzed the results. R. C. H. and B. B. conceived of the idea and wrote the paper with X. Y.

**References**

1. Caux, P. Y., Kent, R. A., Fan, G. T., and Stephenson, G. L. (1996) Environmental fate and effects of chlorothalonil: a Canadian perspective. Crit. Rev. Environ. Sci. Technol. 26, 45–93 CrossRef
2. Sakkas, V. A., Lambropoulou, D. A., and Albanis, T. A. (2002) Study of chlorothalonil photodegradation in natural waters and in the presence of humic substances. *Chemosphere* 48, 939–945 CrossRef Medline

3. Mozzachio, A. M., Rusiecki, J. A., Hoppin, I. A., Mahajan, R., Patel, R., Beane-Freeman, L., and Alavanja, M. C. (2008) Chlorothalonil exposure and cancer incidence among pesticide applicator participants in the agricultural health study. *Environ. Res.* 108, 400–403 CrossRef Medline

4. Carlo-Rojas, Z., Bello-Mendoza, R., Figueroa, M. S., and Sokolov, M. (2004) Chlorothalonil degradation under anaerobic conditions in an agricultural tropical soil. *Water Air Soil Pollution* 151, 397–409 CrossRef Medline

5. Kwon, J. W., and Armbrust, K. L. (2006) Degradation of chlorothalonil in irradiated water/sediment systems. *J. Agric. Food Chem.* 54, 3651–3657 CrossRef Medline

6. Vickers, A. E., Sloop, T. C., and Lucier, G. W. (1985) Mechanism of action of toxic halogenated aromatics. *Environ. Health Perspect.* 59, 121–128 CrossRef Medline

7. Wang, G., Li, R., Li, S., and Jiang, J. (2010) A novel hydrolytic dehalogenase for the chlorinated aromatic compound chlorothalonil. *J. Bacteriol.* 192, 2737–2745 CrossRef Medline

8. Viciu, M. S., Grasa, G. A., and Nolan, S. P. (2001) Catalytic dehalogenation of aryl halides mediated by a palladium/imidazolium salt system. *Organometallics* 20, 3607–3612 CrossRef Medline

9. Reddy, G. V., and Gold, M. H. (2000) Degradation of pentachlorophenol by Phanerochaete chrysosphorum: intermediates and reactions involved. *Microbiology* 146, 405–413 CrossRef Medline

10. Bell, S., Mason, J. R., Timmis, K. N., and Pieper, D. H. (1998) Identification of chlorobenzene dioxygenase sequence elements involved in dechlorinization of 1,2,4,5-tetrachlorobenzene. *J. Bacteriol.* 180, 5520–5528 Medline

11. Xun, L., Topp, E., and Orser, C. S. (1992) Purification and characterization of a tetrachloro-p-hydroquinone reductive dehalogenase from a *Flavobacterium* sp. *J. Bacteriol.* 174, 8003–8007 CrossRef Medline

12. Orser, C. S., Dutton, J., Lange, C., Jablonski, P., Xun, L., and Hargis, M. (1993) Characterization of a *Flavobacterium* glutathione S-transferase gene involved reductive dechlorination. *J. Bacteriol.* 175, 2640–2644 CrossRef Medline

13. Yokota, T., Fuse, H., Omori, T., and Minora, Y. (1986) Microbial dehalogenation of haloalkanes mediated by oxygenase or halohydrolase. *Agric. Biol. Chem.* 50, 453–460 CrossRef Medline

14. Leisinger, T., Bader, R., Hermann, R., Schmid-Appert, M., and Vuillemur, S. (1994) Microbes, enzymes and genes involved in dichloromethane utilization. *Biodegradation* 5, 237–248 CrossRef Medline

15. Bunge, M., Adrian, L., Kraus, A., Opel, M., Lorenz, W. G., Andreesen, J. R., Bunge, M., Adrian, L., Kraus, A., Opel, M., Lorenz, W. G., Andreesen, J. R., Kwon, J. W., and Armbrust, K. L. (1993) Crystallographic analysis of the catalytic mechanism of haloacid dehalogenase from *Paracoccus denitrificans*. *J. Am. Chem. Soc.* 115, 9662–9664 CrossRef Medline

16. van der Ploeg, J., van Hall, G., and Janssen, D. B. (1991) Characterization of the haloacid dehalogenase from *Xanthobacter autotrophicus* GJ10 and sequencing of the dhlB gene. *J. Bacteriol.* 173, 7925–7933 CrossRef Medline

17. Verschueren, K. H., Seljée, F., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1993) Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase. *Nature* 363, 693–698 CrossRef Medline

18. Liang, B., Wang, G., Zhao, Y., Chen, K., Li, S., and Jiang, J. (2011) Facilitation of bacterial adaptation to chlorothalonil-contaminated sites by horizontal transfer of the chlorothalonil hydrolytic dehalogenase gene. *Appl. Environ. Microbiol.* 77, 4268–4272 CrossRef Medline

19. Ren, X., Li, H., and Chen, S. (2011) Cloning of the chlorothalonil-degrading gene cluster and evidence of its horizontal transfer. *Curr. Microbiol.* 62, 1068–1073 CrossRef Medline

20. Yue, W., Xiong, M., Li, F., and Wang, G. (2015) The isolation and characterization of the novel chlorothalonil-degrading strain *Paracoccus* sp. XF-3 and the cloning of the chd gene. *J. Bacteriol. Bioeng.* 120, 544–548 CrossRef Medline

21. Chen, H., Wang, H., Wang, T., Huang, S., Zang, X., Li, S., and Jiang, J. (2016) Identification of the metal center of chlorothalonil hydrolytic dehalogenase and enhancement of catalytic efficiency by directed evolution. *Appl. Environ. Biotechnol.* 1, 30–37 CrossRef Medline

22. D’Souza, V. M., Bennett, B., Copik, A. I., and Holz, R. C. (2000) Divalent metal binding properties of the methionyl aminopeptidase from *Escherichia coli*. *Biochemistry* 39, 3817–3826 CrossRef Medline

23. Winzor, D. J., and Sawyer, W. H. (1995) Quantitative Characterization of Ligand Binding, Wiley-Liss, New York

24. Wattersen, S. I., Mitra, S., Swierzcek, S. I., Bennett, B., and Holz, R. C. (2008) Kinetic and spectroscopic analysis of the catalytic role of H79 in the methionine aminopeptidase from *Escherichia coli*. *Biochemistry* 47, 11885–11893 CrossRef Medline

25. Dreyton, C. J., Knuckley, B., Jones, J. E., Lewallen, D. M., and Thompson, P. R. (2014) Mechanistic studies of protein arginine deiminase 2: evidence for a substrate-assisted mechanism. *Biochemistry* 53, 4426–4433 CrossRef Medline

26. Anderson, V. E. (1992) Isotope effects on enzyme-catalyzed reactions. *Curr. Opin. Struct. Biol.* 2, 757–764 CrossRef Medline

27. Jenson, D. L., and Barry, B. A. (2009) Proton-coupled electron transfer in photosystem II: proton inventory of a redox active tyrosine. *J. Am. Chem. Soc.* 131, 10567–10573 CrossRef Medline

28. Mitra, S., and Holz, R. C. (2007) Unraveling the catalytic mechanism of nitrite hydratases. *J. Biol. Chem.* 282, 7397–7404 CrossRef Medline

29. Elrod, J. P., Hogg, J. L., Quinn, D. M., Venkatasubban, K. S., and Schowen, R. L. (1980) Protonic reorganization and substrate structure in catalysis by serine proteases. *J. Am. Chem. Soc.* 102, 3917–3922 CrossRef Medline

30. Chow, C., Xu, H., and Blanchard, J. S. (2013) Kinetic characterization of hydrolysis of nitrocinin, cefoxitin, and meropenem by B-lactamase from *Mycobacterium tuberculosis*. *Biochemistry* 52, 4097–4104 CrossRef Medline

31. Biro, F. N., Zhai, J., Doucette, C. W., and Hingorani, M. M. (2010) Application of stopped-flow kinetics methods to investigate the mechanism of action of a DNA repair protein. *J. Vis. Exp.* 1874 CrossRef Medline

32. Estrella, M. A., and Solomon, E. I. (2008) Isotope effects on enzyme-catalyzed reactions. *Curr. Opin. Struct. Biol.* 18, 385–428 CrossRef Medline

33. Estrella, M. A., and Solomon, E. I. (2008) Isotope effects on acid-base equilibria. *Adv. Enzymol. Relat. Areas Mol. Biol.* 85, 267–372 CrossRef Medline

34. Estrella, M. A., and Solomon, E. I. (2008) Isotope effects on enzyme-catalyzed reactions. *Adv. Enzymol. Relat. Areas Mol. Biol.* 85, 267–372 CrossRef Medline

35. Estrella, M. A., and Solomon, E. I. (2008) Isotope effects on enzyme-catalyzed reactions. *Adv. Enzymol. Relat. Areas Mol. Biol.* 85, 267–372 CrossRef Medline