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Kinetic and Structural Characterization of a *cis*-3-Chloroacrylic Acid Dehalogenase Homologue in *Pseudomonas* sp. UW4: A Potential Step Between Subgroups in the Tautomerase Superfamily

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Abstract

A *Pseudomonas* sp. UW4_01740 (designated Ps01740) protein of unknown function was identified as similar to 4-oxalocrotonate tautomerase (4-OT)-like and *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD)-like subgroups of the tautomerase superfamily (TSF). Ps01740 lacks only Tyr-103 of the amino acids critical for *cis*-CaaD activity (Pro-1, His-28, Arg-70, Arg-73, Tyr-103, Glu-114). As Ps01740 may represent an important variant of these enzymes, its kinetic and structural properties have been determined. Ps01740 shows tautomerase activity with phenylolpyruvate, but lacks native 4-OT activity and dehalogenase activity with the isomers of 3-chloroacrylic acid. Ps01740 shows mostly low-level hydratase activity at pH 7.0, converting 2-oxo-3-pentynoate to acetopyruvate, consistent with *cis*-CaaD-like behavior. At pH 9.0, this compound results primarily in covalent modification of Pro-1, which is consistent with 4-OT-like behavior. These observations could reflect a pK_a for Pro-1 that is closer to that of *cis*-CaaD (~9.2) than to 4-OT (~6.4). A structure of the native enzyme, at 2.6 Å resolution, highlights differences at the active site from those of 4-OT and *cis*-CaaD that add to our understanding of how

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Accession Codes

The atomic coordinates and structure factors have been deposited as Protein Data Base entry 5UIF.

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contemporary TSF reactions and mechanisms may have diverged from a common 4-OT-like ancestor.

Graphical Abstract

Keywords

tautomerase superfamily; 4-oxalocrotonate tautomerase; *cis*-3-chloroacrylic acid dehalogenase; 2-oxo-3-pentynoate; evolution

cis-3-Chloroacrylic acid dehalogenase (*cis*-CaaD) is the namesake of one of five major subgroups in the tautomerase superfamily (TSF).^{1–3} Dehalogenation represents one reaction in a diverse set of reactions catalyzed by TSF members that includes tautomerization,⁴ hydration,⁵ decarboxylation,⁶ aldol condensation,⁷ and oxygenation.⁸ The TSF is best known for two characteristics. First, superfamily members are mostly constructed from a single β – α – β structural unit (58–84 amino acids) or two fused β – α – β units (110–150 amino acids).³ Second, all of the experimentally characterized members (~30) have a catalytic amino-terminal proline (Pro-1) that functions as a general acid or base depending on the pK_a of the prolyl nitrogen.³ Thus far, no characterized reaction in the TSF requires a coenzyme or metal ion for catalysis.

cis-CaaD catalyzes the hydrolytic dehalogenation of *cis*-3-chloroacrylic acid to afford malonate semialdehyde (Scheme 1).^{1,2} The enzyme is part of five-step pathway that converts the nematocide, 1,3-dichloropropene, to acetaldehyde, which is presumably channeled into the Krebs cycle.⁹ The enzyme has been extensively characterized by kinetic, mechanistic, and structural studies.^{1,2,10,11} It is a trimer where each monomer consists of 149 amino acids that code for two fused β – α – β motifs. Six active site amino acids (Pro-1, His-28, Arg-70, Arg-73, Tyr-103, Glu-114) have been implicated in the catalytic mechanism. In the current working mechanistic hypothesis, Glu-114 activates water for attack at C-3. The arginine residues, Arg-70 and Arg-73, interact with the C-1 carboxylate group (to bind and polarize the substrate), and Pro-1 functions as a general acid and provides a proton at C-2. His-28 could be involved in the binding of the carboxylate group and Tyr-103 assists Glu-114 in the activation of the water molecule.

There has been much speculation about the evolutionary history of the TSF.^{3,12,13} The predominance of TSF members with a single β – α – β unit or two fused β – α – β units has long suggested that a gene fusion event (or events) took place followed by the diversification of function that is seen today. To gain a deeper understanding of the relationships across the

superfamily, a large-scale analysis comparing all known protein sequences of the TSF was generated.¹⁴ Although its function is not known, one of the sequences, *Pseudomonas* sp. UW4_01740 (designated Ps01740), showed significant similarity to both the 4-oxalocrotonate tautomerase (4-OT)-like and *cis*-CaaD-like subgroups of the superfamily.

This protein and two close homologs are more “*cis*-CaaD-like” than “4-OT-like” as they lack only Tyr-103, a residue that is required for function in *cis*-CaaD, but not conserved in the 4-OT subgroup of sequences.^{1–3} Similarly, another protein closest to the 4-OT subgroup consists of a single sequence that is more “4-OT-like”, lacking only Phe-50 among the set of amino acids implicated in the 4-OT mechanism (Pro-1, Arg-11, Arg-39, Phe-50).^{15–19} These observations raise many questions about the kinetic, mechanistic, structural, and biological properties of the enzymes that link the characterized 4-OT and *cis*-CaaD subgroups. As the first step in addressing these questions, one gene sequence for Ps01740, was cloned and the enzyme expressed. Kinetic analysis shows that the enzyme does not have native 4-OT activity (with 2-hydroxymuconate) or dehalogenase activity (with either isomer of 3-chloroacrylate). However, Ps01740 shows tautomerase activity with phenylenolpyruvate (Scheme 2A). It has a low-level hydratase activity (predominately at pH 7.0), converting 2-oxo-3-pentynoate to acetopyruvate (Scheme 2B).^{5,20} At pH 9.0, the enzyme is inactivated by covalent modification of the Pro-1.^{21,22} These observations are consistent with the idea that Ps01740 may represent a potential transition “intermediate” between subgroups. A structure of the native enzyme puts these observations into a structural context.

EXPERIMENTAL PROCEDURES

Materials

Chemicals, biochemicals, and buffers were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific, Inc. (Pittsburgh, PA), EMD Millipore, Inc. (Billerica, MA) or Bio-Rad Laboratories, Inc. (Hercules, CA), unless otherwise noted below. Phenylenolpyruvate (PP) was purchased from Fluka Chemical Corp. (Milwaukee, WI) as the crystalline free acid, which exists in the enol form. 2-Oxo-3-pentynoate (2-OP) was synthesized, as described elsewhere.²¹ The compounds in the substrate library were obtained from sources reported elsewhere.²³ The DEAE-Sepharose fast flow resin and the PD-10 Sephadex G-25M buffer-exchange columns were obtained from GE Healthcare Bio-Sciences (Pittsburgh, PA). The Econo-Column chromatography columns (used for the DEAE-Sepharose columns) were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). The Amicon stirred cell concentrators and the ultrafiltration membranes (10000 Da, MW cutoff) were purchased from EMD Millipore Inc. (Billerica, MA). InstantBlue for staining sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels was obtained from C.B.S. Scientific Company, Inc. (Del Mar, CA).

Bacterial Strains, Plasmids, and Growth Conditions

Escherichia coli strain BL21-Gold(DE3) was obtained from Agilent Technologies (Santa Clara, CA). The gene encoding *Pseudomonas* sp. UW4_01740 (designated Ps01740) was codon-optimized for expression in *E. coli*, synthesized, and cloned into the expression vector pJ411 by DNA2.0, Inc. (now ATUM, Newark, CA). Cells were grown in Luria–Bertani (LB)

media, supplemented with 25 mM Na₂HPO₄ and 25 mM KH₂PO₄, buffered to pH ~6.75, 5 mM Na₂SO₄, 2 mM MgSO₄, and kanamycin (Kn, 30 µg/mL).

General Methods

Steady-state kinetic assays were performed on an Agilent 8453 diode-array spectrophotometer at 22 °C. Nonlinear regression data analysis was performed using the program Grafit (Erithacus Software Ltd., Staines, U.K.). Protein concentrations were determined by the Waddell method.²⁴ SDS-PAGE was carried out on denaturing gels containing 12% polyacrylamide.²⁵ Electrospray ionization mass spectrometry was performed on an LCQ electrospray ion-trap mass spectrometer (Thermo, San Jose, CA), housed in the ICMB Protein and Metabolite Analysis Facility at the University of Texas at Austin. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian INOVA-500 or a Varian DirectDrive 600 MHz spectrometer (Palo Alto, CA). NMR signals were analyzed using the software program SpinWorks 3.1.6 (Copyright © 2009 Kirk Marat, University of Manitoba).

Expression and purification of Ps01740

Fresh BL21Gold(DE3) transformants containing the pJ411-Ps01740 expression vector were collected from a LB/Kn plate and used to inoculate a preculture (LB/Kn medium, 10 mL). After growth for ~6 h at 37 °C, the preculture was used to inoculate 1 L of the supplemented LB media. After overnight growth at 37 °C with shaking (250 rpm), the culture was incubated at 22 °C for 1 h with shaking, and then made 0.5 mM in isopropyl-β-D-thiogalactoside to induce expression. After 4 h at 22 °C with shaking, the cells were harvested by centrifugation (10 min at 2500g) and resuspended in 10 mM Na₂HPO₄ buffer (pH 7.3, buffer A) to a total volume of about 15 mL. The cell suspension was chilled on ice for 15 min, after which the cells were disrupted by sonication for 3 min at 50% duty cycle/50% output using a Vibra-Cell sonicator model VC250B (Sonics & Materials, Inc. Danbury, CT). Unbroken cells and debris were removed by centrifugation (45 min at 18000g). The clear supernatant was applied to a DEAE-Sepharose column (~15 mL bed volume), which had been equilibrated in buffer A. The column was washed with buffer A (3 × 15 mL), and retained proteins were eluted with a linear gradient of buffer A made 500 mM in NaCl. [Typical purification conditions for TSF members use (NH₄)₂SO₄.^{17–19,23} However, under these conditions, the flocculation occurs. Hence, NaCl was used.] The protein eluted ca. 150 mM NaCl. The fractions from the DEAE-Sepharose column containing PputUW4_01740 were pooled, and crystalline NaCl was added to increase its concentration by 2M, resulting in a final NaCl concentration of ~2.15 M. During incubation on ice for 3 h, PputUW4_01740 slowly forms a crystalline-like precipitate. The precipitate was collected by centrifugation (15 min at 18000g), and washed by resuspending the precipitate with 30 mL of buffer A made 2.15 M in NaCl. The resuspended, precipitated protein was pelleted by centrifugation as described above, and the clear supernatant was removed. After repeating this washing step twice, 30 mL of 10 mM NaH₂PO₄ buffer (pH 7.3) was added to the protein pellet, after which the protein readily dissolved. The resulting clear solution of PputUW4_01740 was concentrated to ~3 mL using an Amicon concentrator equipped with a 10 kDa cutoff membrane-filter. Traces of NaCl were removed using a PD-10 gel filtration column, which had been equilibrated in buffer A. Protein was eluted

with buffer A, and concentrated to ~20 mg/mL using an Amicon concentrator. SDS-PAGE analysis indicated that the protein was purified to near homogeneity. Aliquots were flash-frozen in liquid nitrogen and stored at -80 °C until further use.

Steady State Kinetics

A 100 mM stock solution of phenylenolpyruvate (PP) was prepared by dissolving the appropriate amount of the crystalline free acid in absolute ethanol. Ps01740 was diluted to the assay concentration (0.23 μ M) and incubated for 1 h before use. The ketonization of PP was monitored by following the decrease in absorbance at 288 nm ($\epsilon = 14,000 \text{ M}^{-1} \text{ cm}^{-1}$) using substrate concentrations ranging from 60–1430 μ M.²³ The other potential substrates [2-hydroxyomuconate, 5-(methyl)-2-hydroxyomuconate, 5-(carboxymethyl)-2-hydroxyomuconate, 2-hydroxy-2,4-pentadienoate, and propiolate] were made up and the reactions followed as described elsewhere.²³ All assays were carried out at 22 °C in 20 mM Na_2HPO_4 buffer (pH 9.0).

¹H NMR spectroscopic monitoring of the incubation mixture containing Ps01740 and *cis*-3-chloroacrylic acid

The ¹H NMR experiments were carried out as previously reported with the following modifications.²⁶ Accordingly, an aliquot (0.6 mL) of *cis*- or *trans*-3-chloroacrylic acid was transferred to an NMR tube from a stock solution made up in 100 mM Na_2HPO_4 buffer. The aliquot contained 4 mg (0.04 mmol) of the substrate. Dimethyl sulfoxide-*d*₆ (DMSO-*d*₆, 30 μ L) was also added to the tube. The pH of the reaction mixture was then adjusted to 9.3 using small amounts of an aqueous 1 M NaOH solution. Subsequently, Ps01740 or 4-OT (1 mg) was added to the reaction mixture. ¹H NMR spectra were recorded 7 days after the addition of enzyme to the NMR tube. The chemical shifts for *cis*- and *trans*-3-chloroacrylate, malonate semialdehyde, acetaldehyde, and the hydrates are reported elsewhere.²⁶ The approximate amount of product in the mixtures was determined by integration, as described previously.²⁶

UV/Vis spectroscopic monitoring of the incubation mixture containing Ps01740 and 2-oxo-3-pentynoate

A stock solution of 2-oxo-3-pentynoate (2-OP) was prepared by dissolving 4 mg of the free acid in 100 mM Na_2HPO_4 buffer (600 μ L) to give a final concentration of 59 mM (pH ~7). The enzyme was diluted from a concentrated stock solution (8.5 mg/mL) to 80 μ g/mL in 50 mM NaH_2PO_4 buffer (5.8 μ M), pH 7.0 or 9.0. 2-OP was added to a final concentration of 300 μ M in a quartz cuvette (10 mm path length), and spectra were recorded every 3 min. The rate of formation of acetopyruvate was monitored at 294 nm ($\epsilon = 7,000 \text{ M}^{-1} \text{ cm}^{-1}$) in 50 mM NaH_2PO_4 buffer, pH 9.0.⁵ The concentration of 2-OP varied between 60–1900 μ M.

Irreversible inactivation of Ps01740 by 2-oxo-3-pentynoate and mass spectrometric analysis

The stock solution of 2-OP was prepared, as described above. Ps01740 was diluted from the same concentrated stock solution (above) to 1 mg/mL in 50 mM NaH_2PO_4 buffer (pH 7.3 and 9.5). 2-OP was added to a final concentration of 6 mM (final pH 7.0 and 9.0), and the

reaction mixture was incubated at 22 °C for 15 min. The reaction mixture was made 50 mM in NaBH₄ (from a 1M stock solution in ultrapure water) and incubated at 22 °C for 1 h. The reaction mixture was then flash-frozen in liquid nitrogen and stored at –80 °C. Sample preparation for ESI-MS analysis was performed as described elsewhere.⁵

Peptide Mapping and MALDI Mass Spectrometry (MALDI-MS) Analysis

The enzyme was inactivated by 2-OP (at pH 9.0) and reduced with NaBH₄, as described above. Subsequently, the mixture was desalted using a PD-10 Sephadex G-25 column, and treated with a protease V-8 solution, as described elsewhere.²³ The resulting peptide mixtures were analysed by MALDI-MS, as described previously.²³

Crystallization of Ps01740

Initial crystallization conditions were screened in sparse-matrix format using a Phoenix crystallization robotic system (Art Robbins Instruments, Sunnyvale, CA) with a protein concentration of 20 mg/mL in 10 mM NaH₂PO₄ buffer at pH 7.3. The conditions that identified initial hits were then optimized using the sitting drop with vapor-diffusion method. The crystals for Ps01740 were grown in crystallization solution containing 0.1 M CHES buffer at pH 9.0 and 18% PEG 8000 at room temperature. The crystals of Ps01740 was cryoprotected in mother liquor containing 20% glycerol, prior to exposure to X-ray.

Data collection, processing, structure determination, and refinement

All X-ray data were collected at the Advanced Light Source beamline 5.0.3 (ALS, Berkeley, CA). The X-ray dataset for the native Ps01740 was collected at a wavelength of 0.97648 Å at 100 K. The data sets were indexed, integrated, and scaled using HKL2000.²⁷ The data collection statistics are summarized in Table 2.

The structure for Ps01740 was determined by molecular replacement (MR) using Phaser-MR²⁸ and Autobuild²⁹ from the PHENIX suite of programs. The Cg10062 structure (PDB entry 3N4G, 26% sequence identity) was used as a search model for the initial phase estimates for Ps01740 structure factors. Structure refinement was performed using Phenix Refine,³⁰ in which TLS parameter refinement was included in the refinement.³¹ Model structures were evaluated during and after refinement using Molprobity.³² The refinement statistics for the structures are summarized in Table 2. All figures were prepared with PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).³³

Light scattering experiments

The light scattering experiments were carried out at 25 °C on a DAWN HELEOS-II Multi-angle light scattering photometer with an Optilab T-rex refractometer detector and a Wyatt QELS dynamic light scattering detector (MALS-QELS system) (Wyatt Technology, Santa Barbara, CA), as described elsewhere.³⁴ Samples were delivered through a TSK-GEL G300PW_{XL} size-exclusion column (7.8 × 300 mm, pore size 300 Å) (Tosoh Bioscience LLC, King of Prussia, PA) connected to a Shimadzu LC-20AD HPLC System (Model WTC-030S5). The solvent was 50 mM NaH₂PO₄ buffer, pH 7.0, and the flow rate was 0.4 mL/min.³⁴ Molar mass moments in g/mole were determined with 20 µL samples at 25 and

2.5 μ M. Molar masses, peak concentrations, and hydrodynamic radii were determined with Astra 6 software (Wyatt Technology).

RESULTS AND DISCUSSION

The *cis*-CaaD homologue, Ps01740, was overproduced in *E. coli* BL21(DE3) and purified to near homogeneity. The typical yield was about 100 mg of pure protein per liter of culture. ESI-MS analysis of the purified protein showed a single major signal corresponding to a monoisotopic mass of 13528 Da (calc. 13659 Da) in the reconstructed mass spectrum. The mass difference of 131 Da between the theoretical and the observed mass indicates that the translation-initiating methionine is post-translationally removed, resulting in a mature protein (127 amino acids) with an N-terminal proline.³⁵

In order to investigate the potential catalytic abilities of Ps01740, the enzyme was screened for activity against a library of compounds that are known substrates for TSF members. This library includes 2-hydroxymuconate, 5-(methyl)-2-hydroxymuconate, 5-(carboxymethyl)-2-hydroxymuconate, 2-hydroxy-2,4-pentadienoate, propiolate, and phenylenolpyruvate (PP).²³ This analysis showed that Ps01740 only processes PP, where the kinetic parameters are shown in Table 1. The kinetic parameters are comparable to those obtained for 4-OT and greater than those obtained for *cis*-CaaD with a 400-fold greater k_{cat} , 2-fold greater K_{m} , and 194-fold greater $k_{\text{cat}}/K_{\text{m}}$.^{36,37}

Ps01740 was also tested for its ability to catalyze the hydrolytic dehalogenation of *cis*- and *trans*-3-chloroacrylate.²⁶ After 7 days, no conversion of either isomer to products could be detected by ¹H NMR spectroscopy. In the same time period, 4-OT converts ~76% of *trans*-3-chloroacrylate to malonate semialdehyde, acetaldehyde, and the hydrates.²⁶ (The lengthy incubation period results in the non-enzymatic decarboxylation of malonate semialdehyde.)

Ps01740 was incubated with 2-oxo-3-pentynoate (2-OP), a well-known probe of the ionization state of Pro-1 in TSF members.^{3,20} In general, if the $\text{p}K_{\text{a}}$ of Pro-1 is low in the TSF member (< 7), it is irreversibly inactivated by the formation of a covalent bond between the prolyl nitrogen of Pro-1 and C-4 of 2-OP at pH ~7.²¹ If the $\text{p}K_{\text{a}}$ of Pro-1 is high (> 9), the enzyme catalyzes a hydration reaction to form acetopyruvate at pH ~7.²⁰ 4-OT and related members are inactivated because Pro-1 has a $\text{p}K_{\text{a}}$ of 6.4.¹⁶ *cis*-CaaD, where the $\text{p}K_{\text{a}}$ of Pro-1 is estimated to be 9.2,³⁸ carries out a hydration reaction.¹

The reactions of Ps01740 and 2-OP were followed at pH 7.0 and 9.0 by UV/Vis spectroscopy for 1 h (Figure 1). At pH 7.0, there was a decrease in absorbance at 234 nm (corresponding to loss of 2-OP) and increases in absorbance at 294 nm (corresponding to the formation of acetopyruvate, Scheme 3, Route A) and 340 nm (corresponding to the inactivation of Ps01740, Scheme 3, Route B) (Figure 1A). The absorbance at 340 nm sharply increases in the initial 6 min and then slowly shifts to a new maximum at 324 nm. At pH 9.0, the decay at 234 nm is not as significant or as fast. The increase at 294 nm is also not as significant or as fast, whereas the initial increase at 340 nm is nearly complete within the first 3 min. As was observed at pH 7, the absorbance at 340 nm at pH 9 slowly shifts to the new maximum at 324 nm.

The development of absorbance maxima at 324 nm and 340 nm upon incubation with 2-OP has been previously observed in similar experiments with another TSF members.¹³ Both signals have been attributed to the formation of the enamine species between the prolyl nitrogen and 2-OP, perhaps due to the different active site environments (Scheme 3, Route B).¹³ Another possibility is that the allene species precedes the enamine and could be responsible for the initial absorbance maximum at 340 nm. The allene species could be stabilized in the active site by the interaction of the negatively charged oxygen with an active site residue. The mechanism is under investigation.

The observation that hydration predominates at pH 7.0, whereas inactivation predominates at pH 9.0, is consistent with mass spectral experiments. At both pH 7.0 and 9.0, Ps01740 is inactivated by 2-OP due to the covalent modification of the prolyl nitrogen of Pro-1. In the absence of 2-OP, the monoisotopic mass of Ps01740 is 13,528 Da. The reconstructed mass spectrum of a labeling reaction performed at pH 7.0, revealed two major signals corresponding to monoisotopic masses of 13528 Da and 13643 Da, respectively, in a ratio of relative abundance of ~5:1. These masses correspond to the native, unmodified protein (13528 Da) and the protein modified by a single covalent adduct of 2-OP after reduction with NaBH₄ (13642 Da). Both masses were also observed when the labeling reaction was performed at pH 9.0. However, the ratio of relative abundance was 1:1. The difference in ratio likely reflects the pH dependence of covalent modification, as was observed spectrophotometrically. This observation suggests that there is more covalent modification of Pro-1 at pH 9.0 (than at pH 7.0), which likely reflects a higher p*K*_a value for the prolyl nitrogen.

MALDI-MS analysis of the peptide mixture resulting from proteolysis (using protease V-8) of the 2-OP-inactivated Ps01740 at pH 9.0 indicates that Pro-1 is the site of covalent modification. The spectrum shows two relevant signals: one signal corresponds to the unmodified peptide Pro-1-Glu-4 (521.26 Da) and the other signal corresponds to same peptide modified after incubation with 2-OP (637.31 Da). The mass difference between these two signals is 116.05 Da, which is consistent with the covalent modification of Ps01740 by 2-OP at Pro-1.

In order to put these observations in a structural context, the structure of native Ps01740 was determined. The protein crystallized in space group *C2* with 3 monomers per asymmetric unit that form a trimer. Light scattering experiments show that the weight-average molar mass of the native protein in 50 mM NaH₂PO₄ buffer (pH 7.0) is 41.9 ± 0.8 kDa, which closely agrees with the calculated mass (40,548 Da) of the trimer (Supporting information, Figure S1). These observations indicate that the trimer observed in the asymmetric unit is equivalent to the biological unit. The crystal structure of Ps01740 shows the typical TSF fold where each monomer of the trimer has two fused β-α-β subunits (Figure 2A).³ The structure has the same secondary and quaternary structure as that of *cis*-CaaD (PDB entry 3MF8),^{2,39} with which it shares 21% sequence identity, and Cg10062 (PDB entry 3N4G), a *cis*-CaaD homologue, with which it shares 26% identity. *cis*-CaaD and Cg10062 share 32% identity.^{9,10,40} The Ps01740 trimer can be superimposed on that of *cis*-CaaD with a root-mean-square deviation (RMSD) of 1.14 Å (Cα) and on that of Cg10062 with a RMSD of 0.975 Å (Cα) (Figure 2B). Finally, a structural superimposition of the monomers with the 4-

OT dimer shows that the major structural differences are in the loops connecting the two β - α - β subunits and the C-termini of the monomers (Figure 2C).

The active site in both Ps01740 (Figure 3A) and *cis*-CaaD (Figure 3B) is a cavity on the surface created by the ends of the β -strands of two neighboring monomers. Sequence analysis identified Pro-1, His-28, Arg-68, Arg-71, and Glu-112 as the active site residues in Ps01740 that correspond to five of the six critical ones for activity in *cis*-CaaD (Pro-1, His-28, Arg-71, Arg-73, Glu-114). Ala-101' in Ps01740 takes the place of Tyr-103' in *cis*-CaaD (where the prime refers to an adjacent subunit). Examination of the Ps01740 active site shows an interaction between the η -nitrogen of Arg-68 and the carboxylate oxygen of Glu-112 (2.2 Å) (Figure 3A). In addition, the missing side chain of Tyr-103 creates a channel in the active site that leads to the center of the trimer. A potential substrate for Ps01740 could extend into this channel or the channel could provide a conduit for water molecules or other unknown reactant molecules. Like *cis*-CaaD, the two arginine residues (Arg-71 and Arg-68) are located on one side of Pro-1 in Ps01740. Hence, Ps01740 can process the monocarboxylate substrate, phenylolpyruvate, but not the dicarboxylate substrate, 2-hydroxymuconate. In 4-OT, Pro-1 is flanked by arginine residues (Arg-11' and Arg-39'') to accommodate 2-hydroxymuconate.

Like 4-OT, Ps01740 is also inactivated by 2-OP (by covalent modification of the prolyl nitrogen). Analysis of a Ps01740 crystal incubated with 2-OP shows the appearance of positive density close to Pro-1, consistent with adduct formation between Ps01740 and 2-OP. However, incorporation of the adduct greatly deteriorates the quality of the crystal and reduces the resolution of diffraction. Hence, we cannot report a structure for Ps01740 inactivated by 2-OP at this time.

Although Ps01740 is a contemporary protein, it shows intermediate or “transitional” properties between the *cis*-CaaD and 4-OT subgroups, and perhaps suggests steps in the evolution of proteins in the *cis*-CaaD subgroup from a 4-OT-like ancestor. Kinetic analysis shows a loss of native 4-OT activity (using 2-hydroxymuconate), but tautomerase activity with phenylolpyruvate. A structural basis for this observation is provided by the structure of the native enzyme. Two arginines (Arg-71 and Arg-68) are located on one side of Pro-1 in Ps01740 (like *cis*-CaaD), whereas they flank Pro-1 in 4-OT. In addition, the pK_a of Pro1 is in between that of 4-OT (~6.4) and *cis*-CaaD (~9.2), as reflected by its behavior with the pK_a probe, 2-oxo-3-pentynoate. This work sets the stage for an investigation into other enzymes that link the two subgroups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

<i>cis</i>-CaaD	<i>cis</i> -3-chloroacrylic acid dehalogenase
CHES	<i>N</i> -Cyclohexyl-2-aminoethanesulfonic acid
DEAE	diethyl-aminoethyl
LB	Luria–Bertani
Kn	kanamycin
MALDI	matrix-assisted laser desorption/ionization
MR	molecular replacement
NMR	nuclear magnetic resonance
4-OT	4-oxalocrotonate tautomerase
2-OP	2-oxo-3-pentynoate
PP	phenylenolpyruvate
PEG	polyethylene glycol
PDB	Protein Data Bank
Ps01740	<i>Pseudomonas</i> sp. UW4_01740
RMSD	root-mean-square deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TSF	tautomerase superfamily

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Highlights

Ps01740 has kinetic, mechanistic, and structural features of both 4-OT and *cis*-CaaD

The pK_a of Pro-1 in Ps01740 is between that of 4-OT and *cis*-CaaD

The contemporary enzyme Ps01740 might shed light on the evolution of *cis*-CaaD from a 4-OT-like progenitor

Figure 1.

UV-VIS spectra monitoring the reaction of Ps01740 with 2-OP (final concentration 300 μ M) in 50 mM Na_2HPO_4 buffer at (A) pH 7.0 and (B) pH 9.0. Spectra were recorded every 3 min. The decrease in absorbance at 234 nm corresponds to the loss of 2-OP and the increases in absorbance at 294 nm, 324 nm, and 340 nm correspond to the formation of acetopyruvate and the proposed enamine- and allene-inactivated Ps01740, respectively. At pH 7.0, the hydration of 2-OP predominates whereas at pH 9.0, the inactivation of Ps01740 by 2-OP predominates.

Figure 2.

Overview of the native Ps01740 structure at 2.6 Å resolution and comparison with TSF members. A) The Ps01740 structure as viewed from the three-fold axis. The three active sites are identified by the presence of Pro-1 (one per monomer). Each chain contains 127 amino acids and the N-terminal 113 of them are resolved. B) Superpositioning of Ps01740 (sulfur), *cis*-CaaD (marine, PDB entry 3MF8), and Cg10062 (lime, PDB entry 3N4G). The individual *cis*-CaaD monomers were aligned to the Ps01740 structure because only one *cis*-CaaD monomer was present per asymmetric unit. The average RMSD of three alignments for Ps01740 and *cis*-CaaD is 1.14 Å (Ca). The RMSD for the aligned structures of Ps01740 and Cg10062 is 0.975 Å (Ca). C) Superpositioning of Ps01740 (sulfur) and *cis*-CaaD (marine) monomers and the 4-oxalocrotonate tautomerase (grey, PDB entry 1OTF) dimer. The structural differences are most apparent in the loops connecting the β - α - β unit and the C-termini of the monomers and dimer (4-OT). The RMSD for the Ps01740 and *cis*-CaaD dimers is 1.11 Å (Ca) and the RMSD for Ps01740 and the first 4-OT monomer is 1.19 Å (Ca).

Figure 3.

Comparison of the Ps01740 and *cis*-CaaD active sites. (A) The active site of Ps01740 showing Pro-1, His-28, Arg-71, Arg-68, Ala-101', and Glu-112 (counterclockwise from Pro-1) where the prime indicates that the residue come from an adjacent subunit. (B) The active site of *cis*-CaaD showing the corresponding residues (Pro-1, His-28, Arg-73, Arg-70, Tyr-103', and Glu-114). Replacing tyrosine (*cis*-CaaD) with alanine (Ps01740) creates a channel in the active site that leads to the center of the trimer.

Scheme 1.

The 1,3-dichloropropene catabolic pathway showing the conversion of 1,3-dichloropropene to acetaldehyde via *cis*-3-chloroacrylate and malonate semialdehyde.

Scheme 2.

Two reactions catalyzed by Ps01740.

Scheme 3.

Proposed mechanisms for the (A) Ps01740-catalyzed hydration of 2-OP to acetopyruvate (pH 7.0) and (B) inactivation of Ps01740 by 2-OP (pH 9.0). (For clarity of discussion, the proposed λ_{max} values for the allene and enamine species are shown, but these values have not been confirmed.)

Table 1Steady-state kinetic parameters for Ps01740, 4-OT, and *cis*-CaaD using phenylpyruvate^a

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
Ps01740	230 ± 40	80 ± 9	$(3.5 \pm 0.7) \times 10^5$
4-OT ^b	199 ± 23	73 ± 6	$(3.7 \pm 0.5) \times 10^5$
<i>cis</i> -CaaD ^c	110 ± 30	0.20 ± 0.03	$(1.8 \pm 0.6) \times 10^3$

^aThe kinetic parameters were measured using the assay described in the text in 20 mM Na₂HPO₄ buffer (pH 9) at 22 °C.^bThe kinetic parameters are from reference 36.^cThe kinetic parameters are from reference 37.

Table 2

Data Collection and Refinement Statistics.

Data Collection	Ps01740
space group	<i>C</i> 2
cell dimensions	
a, b, c (Å)	80.7, 58.9, 84.8
α, β, γ (deg)	90.0, 110.4, 90.0
resolution (Å)	50.00 - 2.58 (2.67–2.58) ^{<i>I</i>}
R _{sym} (%) (linear R factor)	0.118 (0.524) ^{<i>I</i>}
<i>I</i> /σ <i>I</i>	7.9 (1.5) ^{<i>I</i>}
completeness (%)	96.0 (76.3) ^{<i>I</i>}
CC _{1/2}	(0.730) ^{<i>I</i>}
CC*	(0.919) ^{<i>I</i>}
Refinement	
resolution (Å)	46.48–2.58
no. of reflections	11402
R _{work} /R _{free} ² (%)	21.92/27.16
no. of atoms	
Protein	2520
Water	57
Average <i>B</i> factor (Å ²)	
Protein	52.3
Water	44.7
R.m.s. deviation	
bond lengths (Å)	0.002
bond angles (deg)	0.464
Ramachandran plot (%)	
residues in most favored regions	99.09
residues in additional allowed regions	0.91
residues in disallowed regions	0.00

^{*I*}Data for the last resolution shell are given in parentheses.

²R_{free} is calculated with 5% of data randomly omitted from refinement.