

Environment-induced conformational and functional changes of L-2-haloacid dehalogenase

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2-Haloacid dehalogenases have been highly studied due to their potential applications in chemical industries and bioremediation. Although biochemical and structural characterizations of the enzyme have been detailed, no information was available regarding environmental effects on the structure–function relationship. Here, circular dichroism spectroscopy (CD) was used to investigate the correlation between changes on the conformation and the function of L-2-haloacid dehalogenase (HadL AJ1) from the *Pseudomonas putida* induced by the environmental factors. Decreased α -helix and increased β -sheet contents were observed in the structure of HadL AJ1 along with activity losses caused by pH, temperature and inhibitors. Regardless of which factor above-mentioned existed, more than 65.0% of HadL AJ1 activity could be remained if its α -helix content was over 12.0%. The maintenance of α -helical structure in HadL AJ1 was indispensable to its catalysis, while β -sheet increase restricts its activity. This study revealed the variation of enzymatic activity due to environmental conditions resulting in structural changes monitored by CD, which contributed to rational modification and was instructive for predicting changes of the enzymatic activity during application.

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Dehalogenases are capable of degrading a wide range of halogenated compounds to the corresponding C–OH compounds, for which, the enzymes are fascinating for industrial chemistry, biosynthesis of chiral compounds and environmental remediation (1–7). 2-Haloacid dehalogenases (EC 3.8.1; 2-HADs) catalyze the cleavage of the carbon–halogen bonds in 2-haloalkanoic acids, releasing a halogen atom and producing corresponding 2-hydroxyalkanoic acids. 2-HADs are classified into four groups based their substrate specificities and the configurations of their products: L-2-haloacid dehalogenase (L-DEX), D-2-haloacid dehalogenase (D-DEX), DL-2-haloacid dehalogenase (configuration-inverting) (DL-DEXi), and DL-2-haloacid dehalogenase (configuration-retaining) (DL-DEXr) (8,9). L-DEX specifically acts on L-2-chloropropionate (L-2-CPA) to produce D-lactate (D-LA).

The reaction mechanism of L-DEX has been clearly elucidated through isotope labeling, site-directed mutagenesis and X-ray crystallography (10–12). The nucleophilic Asp residue in the catalytic site acts at the substrate C-2 atom to form an ester intermediate, and then the Asp carboxyl C atom is attacked by an activated water molecule; consequently, the ester bond is cleaved and the free enzyme and the products are released (Fig. 1) (8,13–15). Currently, structures of L-2-haloacid dehalogenases isolated from *Pseudomonas* sp. YL (L-DEX YL) (16), *Xanthobacter autotrophicus* GJ10 (DhIB) (12), *Burkholderia cepacia* MBA4 (DehIVa) (17) and

Sulfolobus tokodaii (DehSft) (18) have been determined experimentally. Each enzyme consists of a core domain and a subdomain except that DhIB has an additional dimerization domain. The active site cavity is located between the two domains. Although their structures are similar, the chemical properties are not identical, e.g., L-DEX YL and DehSft are both thermostable while the other two enzymes are not. Much has been elucidated regarding the biochemical characteristics and structures of these enzymes, but little is known about effects of environmental conditions on its structure and conjunctive enzymatic activities, especially the dynamic changes of structure responding to the continuous environmental changes. The knowledge gleaned from exploring environmental effects on their structure–function relationship contributes to the improved application of the catalytic functions of these enzymes.

Circular dichroism (CD) spectroscopy analyses chiral objects using a system's differential absorption of left and right circularly polarized light (19). The characteristic shape of CD spectra of molecules reflects their chiral geometry and originates (20). Although CD spectroscopic analysis by itself is not sufficient to obtain absolute structural information of the protein, it is an excellent and widely used method for relatively comparing the changes of secondary structure under different conditions, rapidly evaluating folding and binding properties of proteins (21–25).

In this work, CD spectroscopy was used to explore the effects of environmental factors such as pH, temperature and inhibitors on the conformational and functional relationship of L-2-haloacid dehalogenase (HadL AJ1), from *Pseudomonas putida* strain AJ1

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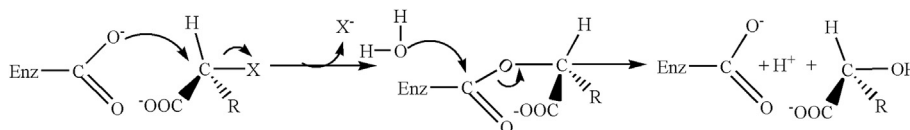


FIG. 1. Reaction mechanism of L-2-haloacid dehalogenase.

(26). Biochemical characterization and conformational studies not only allowed us to better understand the nature of these enzymes but also were necessary to their industrialization. CD spectroscopy provided an option by obtaining the secondary structural information of enzymes to monitor their recycling during the enzymatically catalyzed reactions.

MATERIALS AND METHODS

Plasmid and chemical reagents The DNA sequence of HadL AJ1 from *P. putida* AJ1/23 was obtained from GenBank database (<http://www.ncbi.nlm.nih.gov/nucleotide/151244>). The pET-28a recombinant plasmid was constructed by Invitrogen (Shanghai) and used to transform competent *Escherichia coli* DH5 α cells.

Isopropyl β -D-thio-galactoside (IPTG) was purchased from Shenggong (Shanghai). L-2-chloropropionic acid was purchased from TCI (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). All other chemicals used were analytical grade.

Expression and purification The recombinant protein was expressed in *E. coli* BL21 cells incubated in Luria broth at 37°C. IPTG with a final concentration of 0.5 mM was added to induce for another 4 h when an optical density (at a wavelength of 600 nm) of 0.45–0.55 was reached. The overexpressed enzyme was then purified by nickel affinity chromatography (GE Healthcare) and gel-filtration chromatography using Superdex 200 (HiLoad 16/60, GE Healthcare). The purified protein was stored in 25 mM KH₂PO₄–K₂HPO₄ (pH 8.0). The purity of the recombinant HadL AJ1 was analyzed by SDS-PAGE.

Circular dichroism A Bio-Logic MOS-450 CD spectrometer (Bio-Logic, France) was used to monitor conformational changes to the secondary structure of the protein at 25°C unless otherwise specified. The light source was a xenon lamp, and the slit was 0.8 nm wide. Quartz cuvettes (10 or 1 mm path) were used for measurements, and protein concentrations were 0.0356–0.7138 mg/mL. Data were collected between 185 and 245 nm at a scan rate of 0.5 nm s⁻¹. Buffer scans were subtracted from the enzyme scans and all spectra shown are the average of three individual scans. CD data are reported as the mean residue molar ellipticity ([θ]), which was calculated using a mean residue weight of 26,509.84 Da. The CDpro software (<http://lamar.colostate.edu/~sreeram/CDPro/main.html>) CONTINLL program was used to analyze the CD spectra to determine the α -helix, β -sheet, β -turn and random coil secondary structure fractions using SP43 as a reference set to provide data points between 190 and 240 nm. The α -helix content of the enzyme was chosen as the metric to be used for the estimation of the changes in protein structure. If the variation of α -helix content was 0–9%, 9–18%, 18–36% or 36–100% of the standard which was 17.7%, the enzyme structure was regarded as having undergone no, little, moderate or large changes, respectively.

Enzymatic activity HadL AJ1 activity was assayed using the standard assay system (2) unless stated otherwise. The reaction mixture (1 mL) contained 10 mM L-2-CPA, 100 mM glycine–NaOH (pH 10.0), and enzyme. The reactions were terminated by the addition of 10 μ L of phosphoric acid (85% w/w) following an incubation at 30°C. After the precipitates were removed by centrifugation (14,000 \times g, 10 min), the supernatants were analyzed by HPLC to determine the L-2-CPA contents. One unit of dehalogenase activity was defined as the amount of enzyme that catalyzed 1 μ mol L-2-CPA per min. If the relative activities were 91–109%, 82–91%, 64–82% or 0–50%, the enzymatic activity was regarded as having undergone no, little, moderate or large changes, respectively, unless stated otherwise.

Effects of pH on structure and activity Each of buffers with different pH ranges was used at the concentration of 200 mM. The pH range of 6.0–11.0 was assayed using K₂HPO₄–KH₂PO₄ (pH 6.0–7.5), Tris–H₂SO₄ (pH 8.0–9.5), and Glycine–NaOH (pH 9.5–11.0) to determine the optimum pH for HadL AJ1. The enzyme was incubated at 4°C in the above buffers for 24 h prior to CD measurements and residual activity assays.

Effects of temperature The optimum temperature for the enzyme was determined within the range of 0–60°C. The enzyme was incubated at each tested temperature for 30 min prior to CD measurements and thermal stability analysis.

Effects of inhibitors The structural stability and activity against L-2-CPA were assayed in the presence of 1 mM MnSO₄, Co(NO₃)₂, MgSO₄, ZnSO₄, CuSO₄, Ca(NO₃)₂, HgCl₂ and AgCl. After each of inhibitor was added to the sample for 30 s, CD experiments were performed using the corresponding inhibitor and

buffer without protein as background. Activity assay was performed in the same buffer system as the CD experiments.

RESULTS AND DISCUSSION

Purification The purified enzyme was presented as a single band in SDS-PAGE (Supplementary Fig. S1A) corresponding to a molecular mass of around 26.8 kDa which was in accordance with the theoretical value calculated from the amino acid sequence (26.5 kDa). The molecular mass of the native enzyme was estimated to be 53.8 kDa by gel filtration elution volume (79.65 mL) on a Superdex 200 column (Supplementary Fig. S1B), suggesting that HadL AJ1 existed as a homodimer which was consistent with the majority of earlier reports (2,27–29). In contrast, the enzyme from *P. putida* AJ1 was reported to be predominantly tetrameric with molecular mass of 79.0 kDa and was inactive (26).

HadL AJ1 CD spectra Proteins are divided among four classes, all- α , all- β , α/β and $\alpha+\beta$, based on the shape features of their CD spectra. The α/β and $\alpha+\beta$ class proteins exhibit a positive band in the 190–195 nm region and two negative bands at approximately 208 and 220 nm. The α/β class shows a more pronounced band at 220 nm, while the 208 nm band is more prominent for the $\alpha+\beta$ class (30).

A positive band in the 190–195 nm region and two shoulders at approximately 208 and 220 nm were observed in the CD spectra of HadL AJ1 at different concentrations (Fig. 2). A relatively pronounced shoulder at 220 nm showed that HadL AJ1 was an α/β protein. It has also been confirmed that L-DEX YL, DehIVa and L-HAD_{ST} to which HadL AJ1 showed 60%, 38% and 31% sequence similarity, respectively, were α/β -type hydrolases (Table 1 and Supplementary Fig. S2). Using the CDpro software, the fractions of α -helix, β -sheet, β -turn and random coil in the protein were estimated to be 17.7 \pm 0.7%, 28.2 \pm 0.2%, 21.8 \pm 1.0% and 32.3 \pm 1.2%, respectively. The fractions of α -helix and β -sheet in the L-DEX YL, DehIVa and L-HAD_{ST} calculated from the crystal structure were

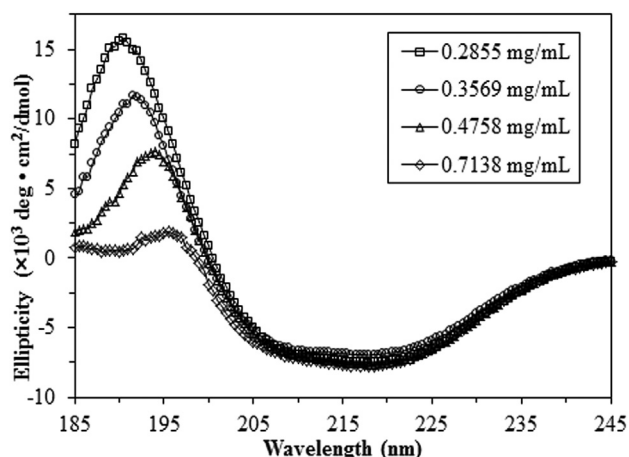


FIG. 2. CD spectra of HadL AJ1. Different concentrations of HadL AJ1 with 0.2855 mg/mL (open squares), 0.3569 mg/mL (open circles), 0.4758 mg/mL (open triangles) and 0.7138 mg/mL (open diamonds) were used.

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