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Mass spectrometric analysis of the reactions catalyzed by L-2-haloacid dehalogenase mutants and implications for the roles of the catalytic amino acid residues

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

L-2-Haloacid dehalogenase catalyzes the hydrolytic dehalogenation of L-2-haloalkanoic acids to produce the corresponding D-2-hydroxyalkanoic acids. Asp10 of L-2-haloacid dehalogenase from *Pseudomonas* sp. YL nucleophilically attacks the α-carbon atom of the substrate to form an ester intermediate, which is subsequently hydrolyzed by an activated water molecule. We previously showed that the replacement of Thr14, Arg41, Ser118, Lys151, Tyr157, Ser175, Asn177, and Asp180 causes significant loss in the enzyme activity, indicating the involvement of these residues in catalysis. In the present study, we tried to determine which process these residues are involved in by monitoring the formation of the ester intermediate by measuring the molecular masses of the mutant enzymes using ionspray mass spectrometry. When the wild-type enzyme and the T14A, S118D, K151R, Y157F, S175A, and N177D mutant enzymes were mixed with the substrate, the ester intermediate was immediately produced. In contrast, the R41K, D180N, and D180A mutants formed the intermediate much more slowly than the wild-type enzyme, indicating that Arg41 and Asp180 participate in the formation of the ester intermediate. This study presents a new method to analyze the roles of amino acid residues in catalysis.

Keywords: L-2-Haloacid dehalogenase; Mass spectrometry; Reaction mechanism

1. Introduction

Dehalogenases catalyze the cleavage of carbon–halogen bonds to decompose organohalogen compounds. Since various organohalogen compounds

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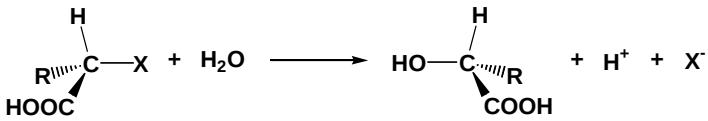

Fig. 1. The reaction catalyzed by L-2-haloacid dehalogenase.

cause environmental pollution, dehalogenases have been attracting a great deal of attention from the viewpoint of environmental technology. Dehalogenases are also useful for the production of chemical compounds from organohalogen compounds as the starting materials.

L-2-Haloacid dehalogenase (L-DEX, E.C. 3.8.1.2) catalyzes the hydrolytic dehalogenation of L-2-haloalkanoic acids to produce the corresponding D-2-hydroxyalkanoic acids (Fig. 1) [1,2]. We isolated L-DEX from *Pseudomonas* sp. YL growing on 2-chloropropionate as the sole source of carbon and energy [3–5]. The enzyme (L-DEX YL) is thermostable, although the source of the enzyme is a mesophilic bacterium. L-DEX YL specifically acts on the L-enantiomers of 2-haloalkanoic acids and is active toward substrates with various carbon chain lengths (C_3–C_{16}) in *n*-heptane. Thus, L-DEX YL is useful for the production of various chiral 2-hydroxyalkanoic acids.

In order to expand the catalytic potential of the enzyme (e.g. alteration of the substrate specificity and improvement of the catalytic efficiency), it is important to clarify the structure and catalytic mechanism of the enzyme. We analyzed the reaction mechanism of L-DEX YL by site-directed mutagenesis, mass spectrometric analysis, and X-ray crystallographic analysis and found that Asp10 of the enzyme nucleophilically attacks the α-carbon atom of L-2-haloalkanoic acid to release the halogen atom, resulting in the formation of an ester intermediate (Fig. 2) [6–11]. The ester intermediate is subsequently hydrolyzed to produce D-2-hydroxyalkanoic acid, and the carboxylate group of Asp10 is regenerated. Comprehensive site-directed mutagenesis studies revealed that Thr14, Arg41, Ser118, Lys151, Tyr157, Ser175, Asn177, and Asp180 are also important for the catalytic function of L-DEX YL: mutation of these residues resulted in a significant loss of enzyme activity [6]. We analyzed the crystal structures of the enzyme complexed with various substrates [9,10]. In these complexes, Asp10 was esterified with the dehalogenated moiety of the substrates, allowing us to obtain structural information on the reaction intermediate. All the above residues were located in the vicinity of the substrate-derived moiety. The crystal structures of the intermediates suggested the roles of the active-site residues. For example, the guanidino group of Arg41 was considered to be the acceptor of the halide ion released from the substrate, which was also supported by the crystallographic analysis of another L-DEX from *Xanthobacter autotrophicus* GJ10 [12].

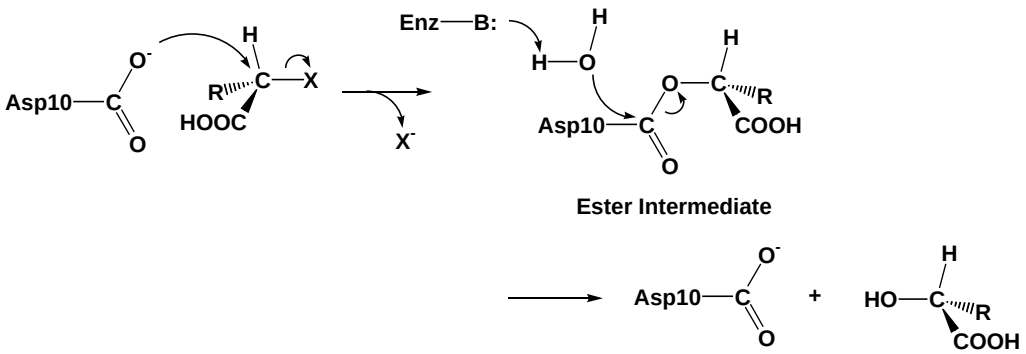

Fig. 2. The reaction mechanism of L-DEX YL.

Although the roles of the active-site residues can be proposed based on the crystallographic data, biochemical data supporting these interpretations are necessary to establish their functions. However, so far, not even which step of the reaction each of the active-site residues is involved in has been determined. In the present study, we tried to identify the roles of the residues by mass spectrometric analysis of the reactions catalyzed by the mutant enzymes. We monitored the time course of the formation of the ester intermediate to see whether the mutation affects this process. As a result, we found that the mutation of Arg41 and Asp180 significantly slowed the formation of the intermediate, indicating their involvement in ester formation.

2. Experimental

2.1. Construction of site-directed mutants

D10A, D10S, T14A, R41K, K151R, Y157F, S175A, N177D, and D180N were prepared as described previously [6]. Synthetic mutagenic primers for S118D, K151A, D180A, and D180E are listed in Table 1. All the mutants were constructed by Kunkel's method [13]. The substitutions were confirmed by determining the nucleotide sequence of the mutated region with a Dye Terminator sequencing kit and an Applied Biosystem 370A DNA sequencer and by measuring the molecular mass with an ionspray mass spectrometer as described below.

2.2. Purification of the enzymes

Recombinant *Escherichia coli* cells producing the mutant enzymes were cultivated at 37 °C for 16–18 h, harvested, disintegrated by ultrasonic oscillation, and purified as described previously [6]. Fractions containing the mutant enzymes were identified by SDS-polyacrylamide gel electrophoresis (PAGE) and measuring the enzyme activity. Protein concentrations were measured with a protein assay kit from Bio-Rad.

Table 1
Oligonucleotide primers for site-directed mutagenesis

S118D (CGA → GTC)	5′-AGCCGTTGTCCAGGATG-3′
K151A (TTT → TGC)	5′-TATCGGGTGCGTAGACC-3′
D180A (GTC → GGC)	5′-CCGTTGCGGCCCACGCG-3′
D180E (GTC → TTC)	5′-CCCGTTGCTTCCCACGC-3′

The mutated triplets are shown in brackets, and the substitutions are underlined.

2.3. Determination of molecular mass by ionspray mass spectrometry

Various L-DEX YL mutants or the wild-type enzyme (10 nmol), 2.5 μmol of L-2-chloropropionate or monochloroacetate, and 2.5 μmol of Tris–H_2SO_4 (pH 9.5) were mixed in a final volume of 100 μl and incubated at 30 °C for the appropriate time. The enzyme incubated without the substrate was used as a control. After incubation, the reaction mixture was immediately injected into a C_{18} column (Puresil 5 μm C18 120 Å, 4.6 mm × 150 mm; Millipore, Tokyo, Japan) and then eluted with a linear gradient of 0–80% acetonitrile in 0.05% trifluoroacetic acid over 40 min at a flow rate of 1 ml/min. The elution was monitored at 215 nm with a UV detector. The eluted fractions were collected and lyophilized. The amino acid sequences of the proteins were determined with a fully automated protein sequencer PPSQ-10 (Shimadzu, Kyoto, Japan). Immediately prior to mass spectrometric analysis, the lyophilized enzymes were dissolved in 100 μl of 50% acetonitrile containing 0.05% acetic acid. The molecular masses of the enzymes were measured in the single quadrupole mode with a PE-Sciex API III mass spectrometer (Sciex, Thornhill, Ont., Canada) equipped with an ionspray ion source. The conditions for the mass spectrometry were the same as those described previously [7,14].

3. Results

3.1. Activities of the mutant enzymes

The wild-type and mutant L-DEX YLs were purified to homogeneity as described in the Section 2. The activities of the enzymes are summarized in Table 2. The mutants without the catalytic nucleophile, D10A and D10S, were absolutely inert, and the activities of all the mutants were significantly lower than that of the wild-type enzyme.

Table 2
Relative activity of the wild-type and mutant L-DEX YLs toward L-2-chloropropionate

Enzyme	k_{cat}/K_m (s^{-1} mM^{-1})[a]	Relative activity (%)[b]
Wild-type	130	100
D10A		0
D10S		0
T14A	0.2	0.34
R41K	0.35	0.68
S118D		0.02
K151A		0.05
K151R	<0.01	0.18
Y157F	3.4	1.8
S175A	0.04	0.37
N177D		0.008
D180A		0.002
D180E		0.06
D180N		0.001

[a] These values are from [6].
[b] The activity was measured by a coupling reaction with D-lactate dehydrogenase as described previously [15]. The amount of NADH produced was quantified.

3.2. Mass spectrometric monitoring of the reactions catalyzed by the wild-type and mutant L-DEX YLs

The molecular masses of the wild-type and various mutant enzymes were determined by ionspray mass spectrometry (Table 3). The measured molecular masses of the enzymes, except for Y157F and S175A, were virtually identical with those calculated based on the amino acid sequences [16]: an error of ±3 Da is acceptable in the analysis of a protein with a molecular mass of about 26 kDa with the mass spectrometer used. The molecular masses of Y157F and S175A were about 513 and 386 Da smaller than the calculated values, respectively. This suggests that the C-terminal five and four amino acid residues of Y157F and S175A, respectively, were removed for unknown reasons.

When the enzymes were incubated with the substrates, the molecular masses of the enzymes except for D10A and D10S increased (Table 3). The increments corresponded to the dechlorinated moiety of L-2-chloropropionate and monochloroacetate, indicating the formation of the ester intermediates. The N-terminal amino acid sequences of the modified

Table 3
Molecular masses of the wild-type and mutant L-DEX YLs

Enzyme	Molecular mass (Da)			
	Predicted[a]	Measured[b]	+CPA[c]	+MCA[d]
Wild-type	26179	26179	26251 (+72)	26238 (+59)
D10A	26135	26135	26134 (−1)	26134 (−1)
D10S	26151	26151	26151 (0)	26151 (0)
T14A	26149	26151	26225 (+74)	26209 (+58)
R41K	26151	26150	26222 (+72)	26209 (+59)
S118D	26207	26205	26279 (+74)	n.d.[e]
K151A	26122	26122	26193 (+71)	n.d.[e]
K151R	26207	26206	26279 (+73)	26264 (+58)
Y157F	26163 (25649[f])	25650	25722 (+72)	n.d.[e]
S175A	26163 (25777[g])	25777	25849 (+72)	25835 (+58)
N177D	26180	26179	26252 (+73)	26239 (+60)
D180A	26135	26133	26206 (+73)	26192 (+59)
D180E	26193	26192	26265 (+73)	26250 (+58)
D180N	26178	26179	26248 (+69)	n.d.[e]

[a] Molecular masses calculated from the amino acid sequence [16].
[b] Molecular masses of the native enzymes determined by mass spectrometric analyses.
[c] Molecular masses of the enzymes incubated with L-2-chloropropionate determined by mass spectrometric analyses.
[d] Molecular masses of the enzymes incubated with monochloroacetate determined by mass spectrometric analyses.
[e] Not determined.
[f] Molecular mass of Y157F lacking the C-terminal five amino acid residues.
[g] Molecular mass of S175A lacking the C-terminal four amino acid residues.

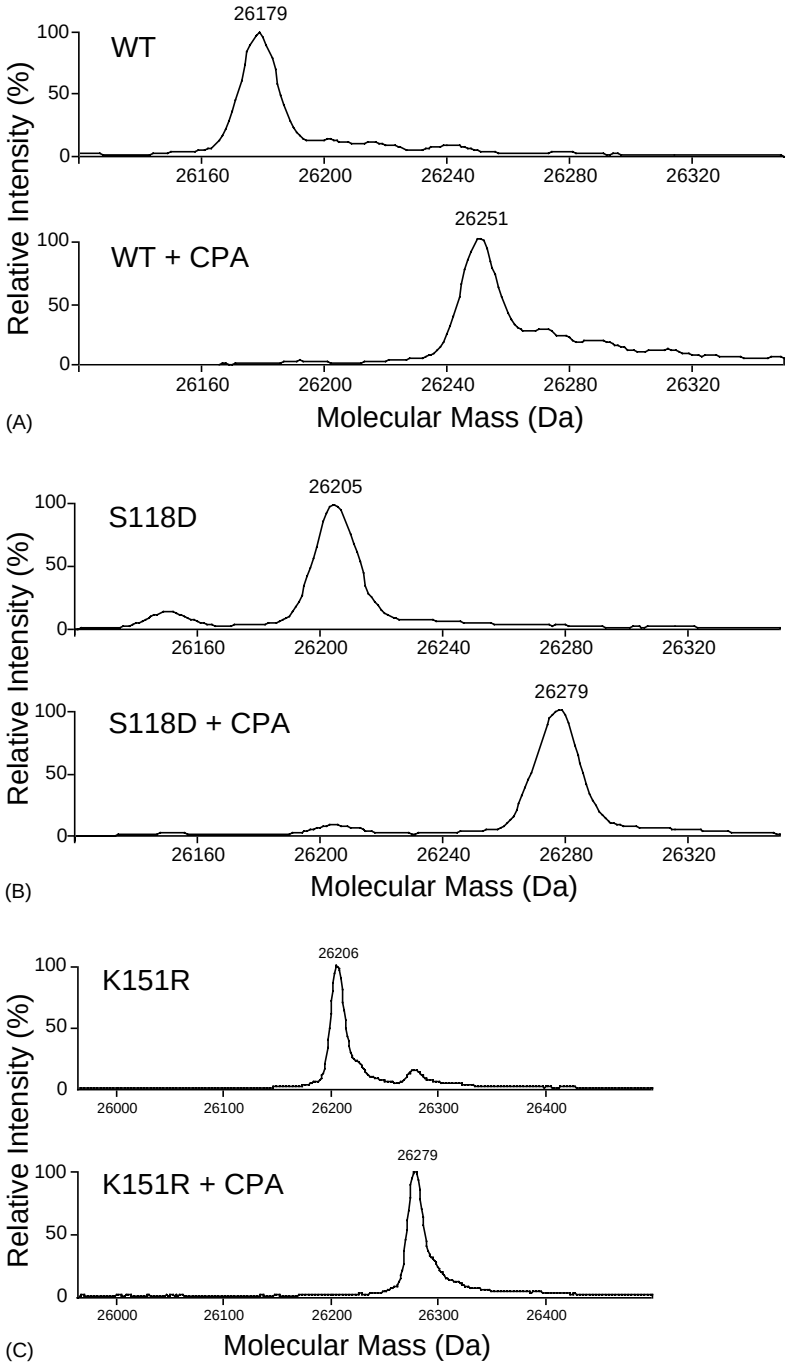

Fig. 3. Mass spectrometric analysis of the wild-type and mutant L-DEX YLs. The mass spectra of the wild-type enzyme (A), S118D (B), K151A (C), S175A (D), and N177D (E) were obtained with an ionspray mass spectrometer as described in the Section 2. The mass spectra of the native enzymes are shown on the upper side, and the spectra of the enzyme incubated with L-2-chloropropionate for 1 min are shown on the lower side.

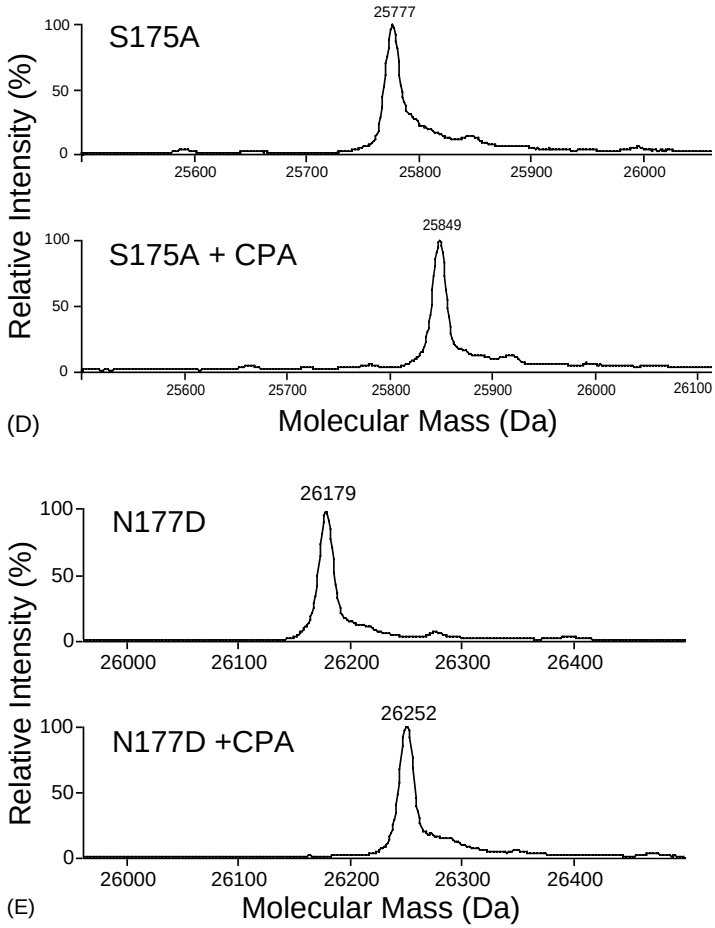

Fig. 3. (*Continued*).

L-DEX YLs were MDYIKGIAF*X*LY, where X is an unidentified residue, whereas the sequences of the unmodified enzymes were MDYIKGIAF*D*LY. These results also support the formation of the ester intermediates.

Fig. 3A shows the mass spectra of the wild-type L-DEX YL incubated with L-2-chloropropionate. After 1 min incubation, virtually all the enzymes were present as ester intermediates. This indicates that the hydrolysis of the intermediate is slower than the formation of the intermediate, resulting in the accumulation of the intermediate. Thus, the rate-limiting step of L-DEX YL is supposed to be the hydrolysis of the ester intermediate.

Although all the mutant enzymes except for D10A and D10S were capable of forming the ester intermediates, the velocities of the intermediate formation were significantly different from one another. As shown in Fig. 3, S118D, K151R, S175A, and N177D were almost totally converted into the ester intermediate after 1 min incubation with the substrate. Similar results were obtained with T14A, K151A, and Y157F (data not shown). In contrast, the mutation of Arg41 and Asp180 significantly delayed the formation of the intermediate (Fig. 4). Even after a 10 min incubation, about half of R41K (Fig. 4A), D180N (Fig. 4B), and D180A (data not shown) remained as the native forms.

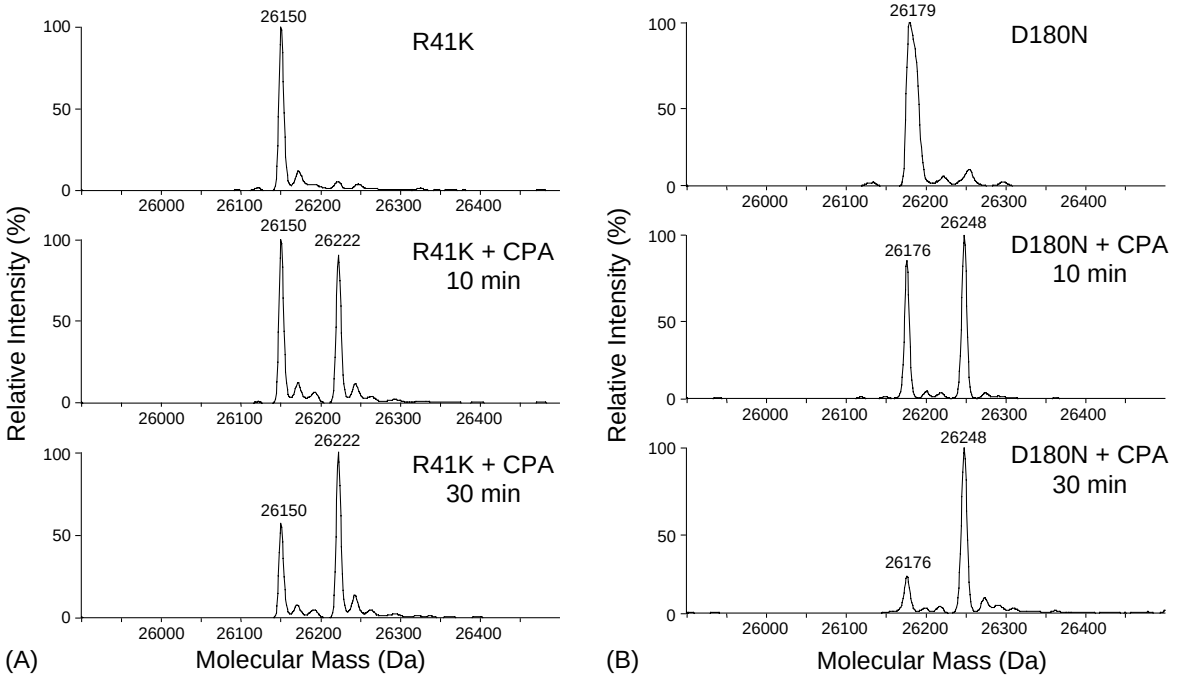

Fig. 4. Mass spectrometric analysis of the R41K (A) and D180N (B) mutant enzymes. The mass spectra of the native enzymes and the enzymes incubated with L-2-chloropropionate for 10 and 30 min are shown.

4. Discussion

We tried to monitor the structural change of L-DEX YL catalyzing the reaction by mass spectrometry. For this purpose, the enzyme reacting with the substrate was introduced into the reversed-phase HPLC column equilibrated with an acidic solvent (pH 2–3) to terminate the reaction immediately and to desalt the enzyme solution. The ester intermediate was stable under this condition, and we succeeded in observing the ester intermediate by ionspray mass spectrometry. The analysis of the wild-type L-DEX YL revealed the accumulation of the ester intermediate in the course of the reaction with L-2-chloropropionate (Fig. 3A). This indicates that the formation of the intermediate is faster than the hydrolysis of the intermediate. Thus, the formation of the ester intermediate is supposed to be the rate-limiting step of the reaction.

We next dissected the roles of the active-site residues. We examined whether the mutation of the active-site residue causes retardation of the formation of the ester intermediate. Fig. 4A clearly shows that the R41K mutant enzyme formed the ester intermediate much more slowly than the wild-type enzyme. This indicates that Arg41 plays a role in the formation of the ester intermediate. We previously proposed that Arg41 is the residue responsible for the abstraction of the halogen atom from the substrate based on the three-dimensional structure of L-DEX YL complexed with various substrates [9,10]. Arg41 is the only positively charged residue that occupies a reasonable position for this function (Fig. 5). In the structure of L-DEX YL complexed with 2-chloropropionamide, we observed an electron density, regarded as a chloride ion released from the substrate, in the vicinity of the guanidino group of Arg41 [9]. The mass spectrometric data presented here support our proposal that Arg41 functions in the formation of the ester intermediate by serving as an acceptor of the halogen atom.

We previously proposed that Asp180 is the base to activate a water molecule for hydrolysis of the intermediate based on the crystallographic analysis of the enzyme [9,10]. However, the present mass spectrometric analysis revealed that the residue is

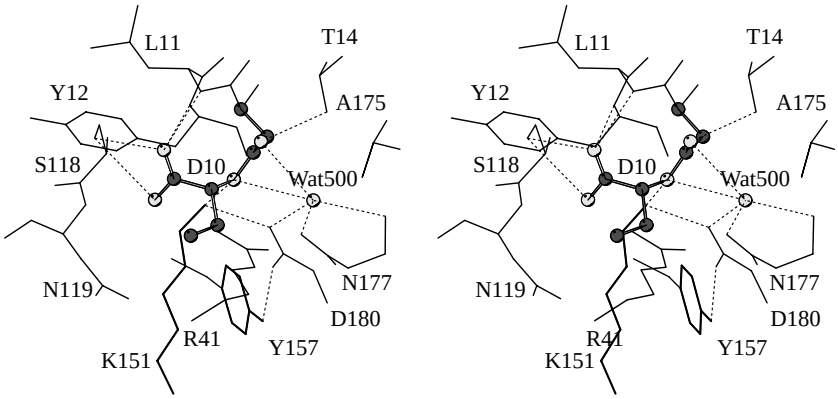

Fig. 5. Stereo view of the active site in the S175A mutant enzyme complexed with L-2-chlorobutyrate. The code for the structure in PDB is 1ZRM. The esterified Asp10 is depicted in ball-and-stick with the carbon and oxygen atoms in black and white spheres, respectively. Hydrogen bonds (2.4–3.4 Å) are shown in dashed lines. The figure was generated with the MOLSCRIPT program [17].

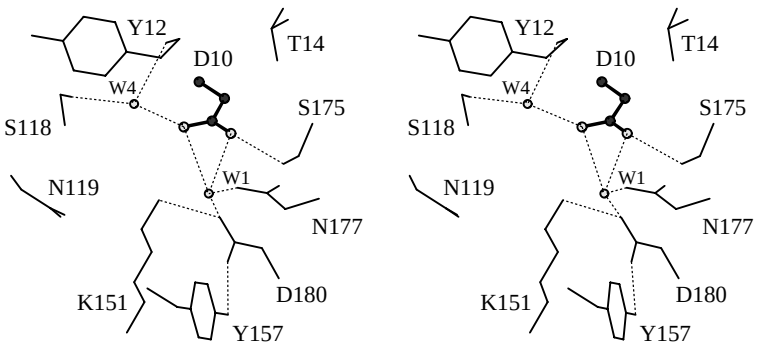

Fig. 6. Stereo view of the active site in the native wild-type L-DEX YL. W1 and W4 stand for water molecules. The code for the structure in PDB is 1JUD. Hydrogen bonds (2.4–3.4 Å) are shown in dashed lines. The figure was generated with the MOLSCRIPT program [17].

also involved in the formation of the ester intermediate; the mutation of Asp180 significantly slowed the formation of the intermediate (Fig. 4B). In the native wild-type enzyme, the $O^{\delta 2}$ atom of Asp180 is associated with the O^{δ} atoms of the catalytic nucleophile Asp10 through a water molecule (W1 in Fig. 6) [18]. This interaction raises the possibility that Asp180 regulates the nucleophilicity of the O^{δ} atoms of Asp10, thereby affecting the formation of the ester intermediate.

In conclusion, we obtained mass spectrometric data showing the involvement of Arg41 and Asp180 in the formation of the ester intermediate. This study provides a novel method to probe the rate-limiting step of the enzyme reaction and to identify the roles of the amino acid residues in catalysis.

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