Novel Catalytic Mechanism of Nucleophilic Substitution by Asparagine Residue Involving Cyanoalanine Intermediate Revealed by Mass Spectrometric Monitoring of an Enzyme Reaction*

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L-2-Haloacid dehalogenase from Pseudomonas sp. YL catalyzes the hydrolytic dehalogenation, in which Asp<sup>10</sup> acts as a nucleophile to attack the α-carbon of L-2-haloalkanoates to form an ester intermediate, which is subsequently hydrolyzed to produce α-2-hydroxyalkanoates. Surprisingly, replacement of the catalytic residue, Asp<sup>10</sup>, by Asn did not result in total inactivation of the enzyme (Kurihara, T., Liu, J-Q., Nardi-Dei, V., Ko-shikawa, H., Esaki, N., and Soda, K. (1995) J. Biochem. 117, 1317–1322). In this study, we monitored the D10N mutant enzyme reaction by ion-spray mass spectrometry, and found that the enzyme shows a unique structural change when it was incubated with the substrate, L-2-chloropropionate. LC/MS and tandem MS/MS analyses revealed that Asn<sup>10</sup> attacks the substrate to form an imidate, and a proton and β-lactic acid are eliminated to produce a nitrile (β-cyanoalanine residue), followed by hydrolysis to reproduce Asn<sup>10</sup>. This is the first report of the function of Asn to catalyze nucleophilic substitution through its conversion to β-cyanoalanine residue as an intermediate structure. Also, these results demonstrate that mass spectrometry is remarkably useful in monitoring enzyme reactions.

Experimental Procedures

Materials—Lysyl endopeptidase of Acromobacter lyticus M497-1 (Lys-C) and phenylisothiocyanate were purchased from Wako Pure Chemical Industries (Osaka, Japan), L-2-chloropropionic acid (L-CPA), L-Asn, L-Asp, and DL-Ala from Nacalai Tesque (Kyoto, Japan), L-2-chloropropionic acid (L-CPA), L-Asp, and DL-Ala from Nacalai Tesque (Kyoto, Japan), β-CNAla from Sigma, and H<sub>2</sub>O (94–97%) from Isotec (Dayton, OH). All other chemicals were of analytical grade.

DNA Techniques—Replacement of amino acid residue(s) was carried out by the method of Kunkel et al. (14). Mutant genes for D10N and L11K were constructed as reported previously (11). The synthetic mutagenic primer designed for the D10N/L11K double mutant was as follows (the underlines indicate the mutated nucleotides): 5′-CAGCGTACCGTACTTGTGAAGGCAATACC. The substitutions were confirmed with a Shimadzu PPSQ-10 protein sequencer (Kyoto, Japan), subsequently hydrolyzed (3, 4). In the x-ray structure, there is a water molecule close to Asp<sup>10</sup>, Ser<sup>775</sup>, Asn<sup>177</sup>, and Asp<sup>180</sup>, the latter three of which are supposed to enhance the nucleophilicity of the water molecule for hydrolysis of the ester intermediate (5) (Scheme 1). These enzymological studies of L-DEX YL have provided critical information on the structures and functions of various hydrolases including P-type ATPases, which have a significant sequence similarity with L-DEX YL and are proposed to have the haloacid dehalogenase fold (6–9). Recent studies on the crystal structure of Ca<sup>2+</sup>-ATPase from sarcoplasmic reticulum revealed that this ATPase has a high three-dimensional structural similarity to that of L-DEX YL and many essential amino acid residues are conserved between these two proteins (10).

In the course of the studies of L-DEX YL, we surprisingly found that replacement of the catalytic residue, Asp<sup>10</sup>, by Asn did not completely inactivate the enzyme, whereas replacement by Ala, Gly, Ser, or Glu resulted in total inactivation (11). One possible explanation is that the activity of the D10N preparation is attributed to the post-translational production of the wild-type enzyme by deamidation of Asn<sup>10</sup>; deamidation of Asn to produce Asp has been shown to occur in a variety of proteins (12, 13). However, it is also possible that the D10N enzyme itself catalyzes the hydrolysis of the substrate.

In the present study, we analyzed the mechanism of dehalogenation catalyzed by the L-DEX YL D10N preparation by ion-spray mass spectrometry (MS), and found that the D10N enzyme itself, in addition to the wild-type enzyme produced by deamidation of the D10N enzyme, catalyzes dehalogenation. Asn<sup>10</sup> was shown to undergo a unique structural change in the course of the catalysis: it was converted into a β-cyanoalanine (β-CNAla) residue via an imidate, and then regenerated in the catalytic cycle. This is the first report of the formation of a β-CNAla residue as an enzyme reaction intermediate. Also, the results demonstrate the validity of mass spectrometry in enzyme dynamics.

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‡ The abbreviations used are: L-DEX YL, L-2-haloacid dehalogenase from Pseudomonas sp. YL; β-CNAla, β-cyanoalanine; HPLC, high performance liquid chromatography; L-CPA, L-2-chloropropionic acid; MS, mass spectrometry; PTH, phenylthiohydantoin.

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an Applied Biosystem 373A DNA sequencer (Foster City, CA), and an ion-spray mass spectrometer PE-Sciex API 300 (Sciex, Thornhill, Ontario, Canada). Mutant enzymes were produced in Escherichia coli JM109.

**Enzyme Purification**—All operations were performed at 4 °C, and 50 mM potassium phosphate (pH 7.5) was used as the standard buffer unless otherwise stated. The recombinant E. coli cells producing the enzyme were grown aerobically at 37 °C for 14 h in LB medium containing 200 μg/ml ampicillin and 0.2 mM isopropyl-1-thio-β-D-galactoside. The cells harvested from a 4-liter culture were disrupted by sonication. The supernatant was fractionated with ammonium sulfate. A fraction of 40–70% saturation was dissolved in the standard buffer, and then applied to a Butyl-Toyopearl 650 column (3 x 25 cm) (TOSOH, Tokyo). The column was washed with 500 ml of the buffer supplemented with 30% (v/v) ammonium sulfate, and the enzyme was eluted with a linear gradient of 30–20% ammonium sulfate in the buffer with a total volume of 1 liter. The fractions containing the mutant enzyme were identified by sodium dodecyl sulfate-polycrylamide gel electrophoresis, dialyzed against 10 mM potassium phosphate (pH 7.5), and applied to a DEAE-Toyopearl 650M column (1.7 x 5 cm) (TOSOH, Tokyo) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 10–50 mM potassium phosphate (pH 7.5) with a total volume of 200 ml. The fractions containing the mutant enzyme were pooled and used as a purified preparation of the mutant enzyme.

**RESULTS**

**Identification of Asp10 and Asn11 as Reaction Intermediates**—The reaction mechanism of L-DEX YL was routinely assayed by determination of chloride ions produced from L-CPA by the method of Iwasaki et al. (15). The standard assay mixture (100 μl) contained 2.5 μmol of L-CPA, 10 μmol of Tris sulfate buffer (pH 9.5), and the enzyme. The reaction was terminated by addition of 10 μl of 1.5 M sulfuric acid after incubation at 30 °C for 10 min. One unit of the enzyme was defined as the amount of enzyme that catalyzes dehalogenation of 1 μmol of L-CPA per min. Protein concentration was determined with a Bio-Rad protein assay kit (Hercules, CA).

**MS Analysis of Wild-Type L-DEX YL, D10N, D10N/L11K, and D10N/D180N Incubated with L-CPA—Wild-type L-DEX YL, the D10N mutant enzyme, the D10N/L11K double mutant enzyme, or the D10N/D180N double mutant enzyme (10 nmol each) was incubated with L-CPA in the standard assay mixture. The reaction was terminated with 10 μl of 20% (v/v) formic acid, followed by centrifugation at 8200 g for 1 min. The enzyme was denoized with Jasco HPLC system (Tokyo) with a C18 reverse phase column (Shiseido Capcell Pak, SG 300 Å 5 μm, 4.6 x 250 mm, Tokyo) at room temperature, employing linear gradients of solvent A (0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid): sample injection; 3 min, 20% B; 12 min, 20–100% B; 3 min, 100% B; 2 min, 100–20% B (flow rate: 1 ml/min). The deionized enzyme was lyophilized, dissolved in 50 μl of 8 M urea in 200 mM Tris sulfate buffer (pH 7.2) with 40% acetonitrile containing 0.1% formic acid at a flow rate of 45 μl/min. The quadrupole was scanned from 300–800 to 2,000 atomic mass units with a step size of 0.1–0.2 atomic mass unit and with a dwell time of 0.2–1 ms/step. The orifice potential was set at 30–60 V. The molecular mass was calculated using the Bio-MultiView software supplied by PE-Sciex.

**LC/MS Analysis of the Peptides Containing Asn10—** The D10N/L11K mutant enzyme incubated and deionized as described above was lyophilized, dissolved in 50 μl of 8 M urea in 200 mM Tris sulfate buffer (pH 7.2), and incubated at 57 °C for 1 h. The enzyme was then digested by addition of 82.5 μmol of Lys-C dissolved in 75 μl of 1 M Tris sulfate buffer (pH 9.0) at 37 °C for 12 h. The proteolysates (55–100 μl) were loaded onto the C18 Shiseido Capcell Pak column connected to the mass spectrometer, PE-Sciex API 300 or API 3000. Elution was carried out with 2% acetonitrile containing 0.1% formic acid for 5 min, followed by a linear gradient of 2–82% acetonitrile containing 0.1% formic acid over 40 min at a flow rate of 40 μl/min. The quadrupole was scanned from 150–200 to 1,000 with a step size of 0.2 and with a dwell time of 0.5 ms/step. The orifice potential was set at 50 V. For detailed analysis of the peptides containing Asn10, the high performance liquid chromatography (HPLC) fractions were collected, concentrated, and injected into the mass spectrometer for tandem MS/MS analysis described below.

**MS/MS Analysis of the Peptides Containing Asn10—** The tandem ion scan mode by introducing the peptides containing Asn10 from Q1 into a collision cell (Q2) and observing the daughter ions in Q3. Q1 was locked on m/z 631.4, 649.5, 653.3, 655.3, 722.6, or 724.5. Q3 was scanned from 10–120 to 700–1,000 with a step size of 0.1 and with a dwell time of 0.5–1.0 ms/step. The orifice potential was set at 40–68 V.

**Reaction of the D10N/L11K Mutant Enzyme with L-CPA in H218O—** The D10N/L11K mutant enzyme (10 nmol) in 17 μl of 50 mM potassium phosphate buffer (pH 7.5) was lyophilized. The lyophilized enzyme was dissolved in 40 μl of H318O containing 2.5 μmol of L-CPA and 10 μmol of Tris sulfate (pH 9.5), and incubated at 30 °C for 60 min. The reaction was terminated by lyophilization. The lyophilized enzyme was denatured with 20 μl of 8 M urea dissolved in 200 μl of Tris sulfate buffer (pH 7.2) prepared with H318O, and digested with Lys-C dissolved in 20 μl of 1 M Tris sulfate buffer (pH 9.0) prepared with H318O at 37 °C for 15 h.

**Identification of Products of Edman Degradation of β-CNαla—** Man Edman degradation of authentic amino acids was carried out (16, 17). Authentic Ala (1.2 mg), Asn (2.6 mg), Asp (1.6 mg), and β-CNαla (1.7 mg) were separately dissolved in 200 μl of 60% pyridine containing 1.5% dimethylformamide, mixed with 20 μl of phenylisothiocyanate, and incubated anaerobically at 45 °C for 40 min. Remaining phenylisothiocyanate was removed with benzene, and the resultant phenylthiocarbamyl derivatives were lyophilized. The lyophilized samples were mixed with 20 μl of trifluoroacetic acid and incubated anaerobically at 45 °C for 15 min. The resultant 2-amino-5-thiazolinone derivatives were extracted with ethyl acetate, dried, and converted to the phenylthiohydantoin (PTH)-derivatives by incubating with 1 N hydrochloric acid at 80 °C for 8 min. The PTH-derivatives were extracted with ethyl acetate, dried, and used for identification and quantification. The dried PTH-derivatives were dissolved in 40% acetonitrile containing 10 mM acetic acid, and loaded onto a reverse phase column for separation of PTH-derivatives (Wakopak W-PTH column, 4.6 x 250 mm) connected to the mass spectrometer, PE-Sciex API 365. Elution was carried out with 40% acetonitrile containing 0.1% formic acid at a flow rate of 46 μl/min. The total ion current chromatogram was recorded in the single-quadrupole mode. The quadrupole was scanned from 200 to 1,000 with a step size of 0.1 and with a dwell time of 0.5 ms/step. The ion-spray voltage was set at 5 kV, and the orifice potential was 50 V.

**RESULTS**

**MS Analysis of Wild-Type L-DEX YL—** We found that structural changes of L-DEX YL in the course of the catalytic reaction can be monitored by MS. The mass spectral changes of the enzyme upon incubation with the substrate L-CPA is shown in Fig. 1. The control enzyme not incubated with L-CPA showed a peak at 26,182 Da (M), which is virtually identical to the value predicted from the nucleotide sequence (26,179 Da): an error of ± 3 Da is acceptable in the analysis of a protein around 26 kDa with the mass spectrometer used. After 4 h of incubation,
the original peak almost disappeared, and a new peak appeared at 26,255 Da (M + 73), which is considered to be an ester intermediate (M + 72) (Scheme 1, I, R = CH₃). The difference between the measured increment (+73) and the estimated one (+72) can be attributed to a measurement error. The accumulation of the ester intermediate indicates that the rate of the ester formation is faster than that of the ester degradation. The original peak (M) increased over a period of 4–20 s and eventually became predominant, while the M + 73 peak decreased and disappeared. This result indicates that most of the substrates had been degraded before this time by the enzyme reaction, and the rate of formation of the ester intermediate thus became slower than the rate of degradation of the intermediate.

Deamidation of D10N—We found that the D10N preparation showed slight dehalogenation activity (relative specific activity, about 1% of that of the wild-type enzyme when determined immediately after purification). The activity increased in a time- and temperature-dependent manner. The D10N preparation showed 3.6 and 6.2% of the wild-type enzyme activity after storage at 4 °C for 2 and 3 months, respectively; when stored at room temperature for about a month, it showed 26% activity of the wild-type enzyme activity. Amino acid sequencing of the D10N preparation showing 26% of the wild-type enzyme activity revealed the occurrence of Asp at the position of Asn₁₀ (data not shown). These results indicate that the side chain amide of Asn₁₀ is slowly deamidated to produce carboxylate.

MS Analysis of D10N—Although the D10N preparation contained the wild-type enzyme produced by the deamidation of Asn₁₀ as described above, its amount in the fresh preparation is considered to be at most 1% of the total enzyme judging from the specific activity of the preparation. Thus it is possible to monitor the structural change of D10N itself by MS, because the small amount of the wild-type enzyme does not interfere with the mass spectra of D10N, which is present abundantly. We determined the molecular masses of D10N incubated with L-CPA for the indicated periods shown in Fig. 2. The control enzyme not incubated with L-CPA showed a peak at 26,180 Da (M), which is virtually identical to the predicted value, 26,178 Da. After 10 s of the incubation, the original peak disappeared, and new peaks appeared at 26,253 Da (M + 73) and 26,162 Da (M − 18). As the relative abundance of the M + 73 species decreased, the M − 18 species increased over a period from 10 s to 1 min. The enzyme occurred predominantly as the M − 18 variant from 20 s to 40 min. The original peak (M) reappeared and increased over a period of 30–60 min, and became predominant by 60 min. When monochloroacetate instead of L-CPA was used as a substrate, a species showing a 60-Da increase was observed instead of the M + 73 species (data not shown), indicating that a covalently linked enzyme-substrate intermediate is produced in the course of the reaction.

Two probable mechanisms can be proposed for the structural change of D10N (Schemes 2 and 3). The first one is shown in Scheme 2: Asn₁₀ attacks L-CPA to form an asparagine β-1-carboxyl ester (II, M + 72), and a proton and α-lactic acid are eliminated from the imidate to produce a nitrile (III, M − 18), which is subsequently hydrolyzed to reproduce Asn₁₀ (M). The difference between the measured (+73) and estimated (+72) mass increments can be attributed to a measurement error or to the conversion of the imidate to an aspartate 1-carboxyl ester as shown in Scheme 4 (V, M + 73) upon denaturation of the enzyme with formic acid (18) (discussed below). In the second probable mechanism shown in Scheme 3, the imidate intermediate is produced in the same manner as shown in Scheme 2, followed by formation of an intramolecular cross-link (IV, M − 18) caused by nucleophilic attack by the hydroxyl group of an amino acid residue positioned in the vicinity of the imidate. The x-ray crystallographic analysis indicates that Ser¹⁷⁵, Ser¹⁷⁶, and Thr¹⁴⁸ possibly play this role (5).

Determination of the Structure Causing the 73-Da Increase—To examine if Asn₁₀ was modified during the incubation with L-CPA, we carried out amino acid sequencing. Native D10N gave the following sequence (the boldface indicates the 10th amino acid residue): Met-Asp-Tyr-Ile-Lys-Gly-Ile-Ala-Asn₁₀-Leu, which is identical to that predicted from the nucleotide sequence. Sequencing analysis of the M + 73 variant gave the following result: Met-Asp-Tyr-Ile-Lys-Gly-Ile-Ala-Asn₁₀-Leu-X, in which X is an amino acid residue whose retention time is different from that of every common amino acid residue including Asn, indicating that Asn₁₀ is modified in the M + 73 variant.

Leu¹¹ of the D10N enzyme was replaced by Lys to create D10N/L11K as described under “Experimental Procedures,” which was used to examine the modification of Asn₁₀ by MS: Lys-C treatment of D10N/L11K produces a short peptide fragment containing Asn₁₀, which is small enough for determination of molecular mass accurately. The mass spectrum of D10N/L11K showed a peak at 26,195 Da (M′), which is virtually identical to the predicted value, 26,193 Da. It was confirmed that the M′ + 73 (26,268 Da) and the M′ − 18 (26,177 Da) variants were formed when D10N/L11K was incubated with L-CPA for 10 s and 8 min, respectively (data not shown). Thus the Lys residue introduced next to the C-terminal side of Asn₁₀ has little effect on the reactivity of the enzyme.

D10N/L11K incubated with (or without) L-CPA was digested with Lys-C, and the resultant peptides were analyzed by LC/
MS. Mass spectrum of a peptide derived from native D10N/L11K showed a peak at m/z 649.5, which was assigned as a positive monovalent ion of the hexapeptide Gly6-Lys11 (Fig. 3A, M'). The hexapeptide isolated from the enzyme incubated with L-CPA for 10 s and denatured with acid showed a new peak at m/z 722.6, which is about 73 Da higher than that derived from the unmodified one (Fig. 3B, M' + 73). To determine the amino acid residue where the mass increment had occurred, tandem MS/MS analysis of the M' + 73 peptide was carried out. The product ions produced from the unmodified and the M' + 73 peptides are shown in Figs. 4, A and B, respectively. The Y series ions at m/z 649.3, 479.0, 408.1, and 261.2 derived from the unmodified peptide were assigned as Gly-Ile-Ala-Phe-Asn-Lys, Ala-Phe-Asn-Lys, Phe-Asn-Lys, and Asn-Lys, respectively (Fig. 4A). The ions produced from the M' + 73 peptide at m/z 722.6, 552.5, 481.2, and 334.4 were about 73 Da higher than the corresponding ions derived from the unmodified one, respectively (Fig. 4B). However, the molecular mass of the fragment ion for the C-terminal Lys derived from the M' + 73 peptide (m/z 147.1) was virtually identical to that from the unmodified one (m/z 147.0). This result indicates that the modification causing the 73-Da increase occurred at Asn10.

Because a 1-Da difference is significant in the mass range shown in Figs. 3 and 4, the exact mass increase in the M' + 73 peptide at Asn10 is 73 Da, not 72 Da. This indicates the presence of the ester, not the imidate, in the peptide. However, this result does not exclude the possibility that an imidate intermediate was actually produced in the reaction, because an imidate is readily hydrolyzed to an ester under a low-pH condition (18) (Scheme 4), which was employed in the present experiment to terminate the enzyme reaction. An 18O atom is expected to be incorporated in the ester intermediate by addition of H218O upon acidification, if the presumed imidate intermediate is indeed hydrolyzed spontaneously by acidification. To examine if the observed ester was produced from the imidate, H218O was added to the reaction mixture at the termination of incubation. The mass spectrum showed a new peak at m/z 724.5 (Fig. 3C, M' + 75), indicating that an oxygen atom of a water molecule was introduced into the hexapeptide 6–11. Tandem MS/MS analysis of the M' + 75 peptide revealed that a 1-Da mass increase occurred at Asn10 (data not shown). No M' + 75 peptide was produced when H216O was added to the reaction mixture at 1 min after the beginning of the reaction (data not shown), indicating that there is no exchange of an 18O atom of H218O with a carbonyl oxygen of the ester bond. These results show that the oxygen atom of a water molecule was incorporated into the peptide at the position corresponding to Asn10 upon denaturation with acid, supporting the view that the actual reaction intermediate is not the ester but the imidate.

Determination of the Structure Causing the 18-Da Mass De-
crease—We carried out the Edman reaction with free $\beta$-CNAla as a substrate, and analyzed the product by MS. It was revealed that the Edman reaction gave PTH-Asn as the most predominant product (data not shown). A nitrile structure undergoes acid-catalyzed nucleophilic addition of water to produce an amide (19). Edman reaction employs strong acids such as trifluoroacetic acid and hydrochloric acid in the reaction cycle. Therefore the nitrile structure of $\beta$-CNAla is converted to an amide in the course of the reaction. Thus, even if a $\beta$-CNAla residue is formed in a peptide, it is converted to PTH-Asn during Edman degradation.

We carried out amino acid sequencing of the M - 18 variant of D10N. It gave the following sequence: Met-Asp-Tyr-Ile-Lys-Leu. Although this result may indicate that Asn$^{10}$ is intact in the M - 18 variant, it is also consistent with the mechanism of $\beta$-CNAla formation at residue number 10 followed by hydrolysis during Edman degradation.

To confirm the $\beta$-CNAla formation, the M - 18 variant of D10N/L11K was digested, and the resultant peptides were analyzed by LC/MS. We obtained a peptide with an m/z value of 631.5 (Fig. 3D, M' - 18), and found by tandem MS/MS analysis that Asn$^{10}$ was specifically modified in such a way as its molecular mass becomes 18 Da lower than the original value (Fig. 4, A and C). Accordingly, the most probable structure formed at residue number 10 in the M - 18 (or M - 18) variant is a $\beta$-CNAla residue. Since nitrile can hardly be produced from the cross-link structure in the process of MS analysis, the mechanism shown in Scheme 3 is excluded.

Reconversion of $\beta$-CNAla into Asn$^{10}$—To examine if the final step of the reaction is the reversion of $\beta$-CNAla to Asn as shown in Scheme 2, D10N/L11K was incubated in H$_2$O in the presence (or absence) of L-CPA for 60 min. If $\beta$-CNAla is reconverted to Asn as shown in Scheme 2, an $\beta$O atom is expected to be incorporated in the side chain amide of Asn$^{10}$ when the mutant enzyme is incubated with the substrate in H$_2$O. After incubation, D10N/L11K was denatured and digested with Lys-C. Since the C-terminal carboxyl group produced by proteolysis contains two $\beta$O atoms when the proteolysis is performed in H$_2$18O (20), the peptide 6-11 recovered from the M' - 18 peptide in H$_2$18O is expected to contain three $\beta$O atoms, two of which are in the carboxyl group of C-terminal Lys and the rest of which is in the side chain amido group of Asn$^{10}$. The hexapeptide isolated from D10N/L11K incubated without L-CPA and digested in H$_2$18O showed a new peak at m/z 653.4, which is about 4 Da higher than that derived from the unmodified one (Fig. 3E, M' + 4). When D10N/L11K was incubated in the presence of L-CPA, the mass spectrum showed a new peak at m/z 655.5, which is 6 Da higher than that derived from the unmodified one (Fig. 3F, M' + 6). We found by tandem MS/MS analysis that two $\beta$O atoms were incorporated in the C-terminal Lys in both the M' + 4 and M' + 6 peptides, and that an $\beta$O atom was incorporated in Asn$^{10}$ specifically in the M' + 6 peptide (data not shown). These results indicate that the nitrile undergoes the nucleophilic attack of a water molecule to produce the side chain amide of Asn$^{10}$.

In conclusion, the unique structural change of D10N occurs through the mechanism shown in Scheme 2: Asn$^{10}$ attacks the substrate to form the imidate, and a proton and $\delta$-lactic acid are eliminated to produce the nitrile. This is the first report showing that Asn functions as a catalytic nucleophile in enzymatic hydrolysis where $\gamma$-cyanoalanine residue is produced as a reaction intermediate. Also, the results demonstrate that mass spectrometry is remarkably useful in monitoring enzyme reactions.

DISCUSSION

Mechanism of the Structural Change of Asn$^{10}$—In the x-ray structure of the L-DEX YL S175A mutant enzyme complexed with various l-2-chloroalkanoates, the carboxyl oxygens of the substrate are hydrogen bonded to the Ser$^{115}$ hydroxyl group and the main chain amido nitrogens of Leu$^{11}$, Tyr$^{12}$, and Asn$^{119}$ (5). The hydrophobic pocket, which is mainly composed of side chains of Tyr$^{12}$, Gly$^{42}$, Leu$^{45}$, Phe$^{69}$, Lys$^{151}$, Asn$^{177}$, and Trp$^{179}$, exists around the alkyd group of the substrate. This pocket possibly plays an important role in stabilizing the alkyd group of the substrate through hydrophobic interactions, and also plays a role in determining the stereospecificity of the enzyme. The guanidino group of Arg$^{41}$ most likely serves as the halogen abstraction site (5). Because none of these amino acid residues were changed in the D10N enzyme, the position of the substrate in the active site of D10N is probably the same as that in the active site of wild-type L-DEX YL. The orientation of the amido oxygen of Asn$^{10}$ is probably similar to that of the carboxyl oxygen of Asp$^{10}$ participating in the ester formation in the wild-type enzyme reaction: it points to the C-terminal protonated amine group of the substrate, whose electrophilicity is probably enhanced by Arg$^{41}$. Thus the side chain amide of Asn$^{10}$ can attack the substrate to form the imidate in the D10N reaction in the same manner as the side chain carboxylate of Asp$^{10}$ does to form the ester intermediate in the wild-type enzyme reaction.

$\delta$-Lactic acid is released from the ester intermediate by hydrolysis in the wild-type enzyme reaction. In contrast, in the D10N enzyme reaction, $\alpha$-$\beta$-elimination of $\delta$-lactic acid and a proton occurred, rather than hydrolysis, as shown in Scheme 2, even though a water molecule to be used for nitrile hydrolysis is probably present in the vicinity of the imidate. Why does the imidate undergo $\alpha$-$\beta$-elimination instead of hydrolysis in the D10N enzyme reaction? There are two possible reasons. The first one is that the electrophilicity of the imido carbon of the imidate is lower than that of the carboxyl carbon of the ester: the nitrogen atom of the imidate is less electron-donating than the oxygen atom of the carbonyl group, making the imido carbon of the imidate less electrophilic. Accordingly, imido carbon is less reactive with the nucleophilic water molecule. The second reason is the presence of a base that abstracts the imidate proton to trigger the $\alpha$-$\beta$-elimination of the imidate. This base is supposed to be Asp$^{10}$, which, in the wild-type enzyme, is suggested to function as a base to activate a water molecule for hydrolysis of the ester intermediate. This speculation is supported by the following observation: the mass spectrum of D10N/D180N incubated with L-CPA for 8 min showed two peaks at 26,177 Da (M' + 2) and 26,251 Da (M' + 4), but no peak was observed at M' - 18 (data not shown). The incubation time (8 min) is long enough for D10N to accumulate as the M - 18 variant. This result suggests that D10N/D180N can form an imidate intermediate, but not a nitrile intermediate, supporting the view that Asp$^{10}$ plays a role in the $\alpha$-$\beta$-elimination of the imidate.

Once the nitrile intermediate is produced, the proton abstracted from the imidate by Asp$^{10}$ is probably transferred to the nitrite nitrogen to enhance the electrophilicity of the nitrite carbon and make Asp$^{10}$ competent to function as a base to activate the water molecule remaining in the vicinity of the nitrite. The activated water molecule is supposed to attack the nitrite carbon to produce an amide.

Formation of free $\beta$-CNAla catalyzed by $\beta$-cyanoalanine synthase (EC 4.4.1.9) has been demonstrated in a wide range of organisms including higher plants (21, 22) and several species of bacteria (23, 24). However, the mechanism of free $\beta$-CNAla synthesis is quite different from that of the $\beta$-CNAla residue formation reported here: free $\beta$-CNAla is synthesized by $\beta$-re-
placement of the mercapto group in cysteine by cyanide (25), whereas the β-CNAla residue is formed by α,β-elimination of the imidate as shown in Scheme 2.

Possible Roles and Occurrence of the β-CNAla Residue in a Protein—Roy et al. (26) have reported that replacement of the side chain amide of Asn⁵ of oxytocin by a nitrile produced an analog with very weak activity, whereas a similar substitution in glycaminide ⁹ produced a highly active analog. Thus replacement of Asn with a β-CNAla residue can alter the functions of a protein. We reported here that the side chain amide of Asn can be converted to a nitrile. These results raise the possibility that an Asn residue is post-translationally converted into a β-CNAla residue in vivo to modify the function of proteins. Although there has been no other report on a naturally produced β-CNAla residue in a protein so far, the presence of a β-CNAla residue has perhaps been unrecognized because of the lack of an appropriate analysis method for amino acid residues constituting proteins: Edman degradation, the most widely used method for amino acid sequencing, results in the conversion of the β-CNAla residue into PTH-Asn, thereby making it impossible to distinguish a β-CNAla residue from an Asn residue. MS analysis of proteins may reveal the presence of a β-CNAla residue in other proteins.

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