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Role of Conserved Histidine Residues in D-Aminoacylase from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6

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D-Aminoacylase from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* A-6 (*Alcaligenes* A-6) was strongly inactivated by diethylpyrocarbonate (DEPC). An H67N mutant was barely active, with a k_{cat}/K_m 6.3 × 10^4 times lower than that of the recombinant wild-type enzyme, while the H67I mutant lost detectable activity. The H67N mutant had almost constant K_m, but greatly decreased k_{cat}. These results suggested that His67 is essential to the catalytic event. Both H69N and H69I mutants were overproduced in the insoluble fraction. The k_{cat}/K_m of H250N mutant was reduced by a factor of 2.5 × 10^4-fold as compared with the wild-type enzyme. No significant difference between H251N mutant and wild-type enzymes in the K_m and k_{cat} was found. The Zn content of H250N mutant was nearly half of that of wild-type enzyme. These results suggest that the His250 residue might be essential to catalysis via Zn binding.

Key words: D-aminoacylase; *Alcaligenes*; histidine residues; Zn enzyme

N-Acyl-D-amino acid amidohydrolases catalyze the hydrolysis of *N*-acyl-D-amino acids to produce the corresponding D-amino acids and fatty acids. Among these enzymes, D-aminoacylase, which reacts with *N*-acyl derivatives of neutral D-amino acids, has been studied in the three genera of *Streptomyces*,[1,2] *Pseudomonas*,[3–5] and *Alcaligenes*.[6–8] While the production of L-amino acids by optical resolution using L-aminoacylase has been intensively studied,[9,10] few studies on the production of D-amino acid using D-aminoacylase have been reported.[11] To develop new D-aminoacylases suitable for D-amino acid production by optical resolution, we screened the microorganisms producing D-aminoacylase in various soils. *Alcaligenes* A-6 was isolated as a bacterium producing D-aminoacylase.[12] *Alcaligenes* A-6 also produces *N*-acyl-D-aspartate amidohydrolase (D-AAase) and *N*-acyl-D-glutamate amidohydrolase (D-AGase), which are specific for *N*-acyl derivatives of D-aspartate and D-glutamate, respectively.[13–15] Besides the availability of the three enzymes from *Alcaligenes* A-6 for the production of D-amino acids, the structure-function relationship (*e.g.* strict substrate specificities) of these enzymes is very interesting. We cloned the three genes encoding these enzymes from *Alcaligenes* A-6 and clarified their primary structures by analyzing their nucleotide sequences.[16–18] On the other hand, the genes encoding L-aminoacylases from porcine kidney and liver, and *Bacillus stearothermophilus*, have been cloned and their sequences have been identified.[19,20] But few studies on the structure and function of L-aminoacylases have been reported.[21,22] Of course, no information is available concerning the structure-function relationship of D-aminoacylase.

The functional importance of a histidine residue in D-AGase from *Pseudomonas* sp. 5f-1 has been confirmed by chemical modification using DEPC, which is a modification reagent specific for histidyl residues.[23] However, the histidine residue responsible for catalysis in D-AGase from *Pseudomonas* sp. 5f-1 has not been identified. In this study, chemical modification with DEPC indicated that the D-aminoacylase from *Alcaligenes* A-6 has histidine residues responsible for catalysis. We found eight conserved histidine residues in the D-aminoacylase, D-AAase, and D-AGase from *Alcaligenes* A-6 by comparing their amino acid sequences. Among them, significant similarity or identity in amino acids up- and down-streams of the histidyl residues in both Asp-X_1-His-X_2-His-Asp-Asp and Ser-His-His-Lys sequences was found. Therefore the histidine residues of conserved Asp-X_1-His^{67}-X_2-His^{69}-Asp-Asp and Ser-His^{250}-His^{251}-Lys sequences of D-aminoacylase from *Alcaligenes* A-6 were individually replaced by asparagine (Asn) or isoleucine (Ile) via

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Abbreviations: DEPC, diethylpyrocarbonate; D-AAase, *N*-acyl-D-aspartate amidohydrolase; D-AGase, *N*-acyl-D-glutamate amidohydrolase; Asn, asparagine; Ile, isoleucine; IPTG, isopropyl-β-D-thiogalactopyranoside; DMSO, dimethyl sulfoxide; SDS-PAGE, sodium dodecyl sulfate poly-acrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetic acid

site-directed mutagenesis. The structural and functional roles of His67, His69, and His250 are discussed.

Materials and Methods

Materials. pUC18, all restriction enzymes, T4 DNA ligase, *Escherichia coli* (*E. coli*) JM109 and agarose type-S were purchased from Nippon Gene. The pKK223-3 expression vector was from Pharmacia. The Taq DyeDeoxy Terminator Cycle Sequencing Kit was from Applied Biosystems, Inc. Primers for mutagenesis and DNA sequencing were from Sawady Technology. *N*-Acetyl-D-leucine, DEPC, and other chemical modification reagents were purchased from Sigma. ExTaq DNA polymerase and M13primer M4 were from Takara Shuzo. All other chemicals were of analytical grade.

Organisms and cultivation. E. coli JM109 was used as a host strain for recombinant plasmids. The plasmid pKNSD2, consisting of a 1.75-kb *Eco*RI-*Hin*dIII fragment of the gene for D-aminoacylase in pKK223-3, was described previously.[24] pANSD2, consisting of the same insert DNA as pKNSD2 in pUC118, was also described in the same report. pKNSD2 was used for the purification of wild-type D-aminoacylase and pANSD2 was for site-directed mutagenesis of the D-aminoacylase gene. Transformants were grown in Luria-Bertani broth containing 50 μg/ml of ampicillin with 0.1 mM of isopropyl β-D-thiogalactopyranoside (IPTG). Plasmids for purification of mutant enzymes were constructed by inserting the mutated fragments into the *Eco*RI-*Hin*dIII sites of pKK223-3.

Assay of enzyme activity. D-Aminoacylase activity was assayed by measuring the hydrolysis of *N*-acetyl-D-leucine as previously described.[16] One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmole of D-leucine per min. Specific activity is expressed as units per milligram of protein. Protein was estimated by the method of Lowry *et al.*

Chemical modification of the enzyme by various site-specific reagents. To examine the amino acid residues responsible for catalytic activity of D-aminoacylase, various site-specific reagents listed in Table 2 were tested for their abilities to inactivate the enzyme. Inactivation reactions were done at 30°C in 0.2-ml reaction mixtures containing 100 mM potassium phosphate buffer, pH 7.0, 0.50 μg of enzyme, and 1.0–10 mM reagents. After 10 min of the chemical modification reaction, samples (50 μl) were removed, and the residual activities were measured by the procedure described above.

Table 1. Oligonucleotides Used in Site-directed Mutagenesis

Mutation	Mutagenic oligonucleotide
His67Asn	5′-TCGTGGGTGTT**T**CGAGTCGATG-3′
His67Ile	5′-GTCGTGGGTG**AT**CGAGTCGATG-3′
His69Asn	5′-GTCGTCGTT**T**GGTGTGCG-3′
His69Ile	5′-GTTGTCGTCG**AT**GGTGTGCG-3′
His250Asn	5′-ACCTTGTGGTT**T**CGAGATCACC-3′
His251Asn	5′-CATGACCTTGTT**T**GTGCGAGATC-3′
MutEco[a]	5′-GATTACGAGTTCCACTTG-3′

The substituted nucleotides are indicated as bold letters. Mutant codons are underlined.

[a] MutEco primer is designed to break the *Eco*RI recognition site upstream of initiation codon ATG (indicated as a broken line).

Site-directed mutagenesis. The primers used for introduction of mutation are listed in Table 1. In the first PCR, two double-stranded DNA fragments were synthesized using plamid pANSD2 as a template. One fragment was synthesized using M13 primer RV and primer for the His-mutant. Another fragment was amplified using M13 primer M4 and MutEco primer for destruction of the *Eco*RI site located in the upstream region of the initiation codon ATG. The reaction mixture for the 1st PCR (50 μl) consisted of ExTaq buffer, 0.2 mM of each dNTP, 2.5 pmol of each primer, 1 ng of template DNA, 3.0% of dimethyl sulfoxide (DMSO), and 2.5 units of ExTaq DNA polymerase. The reaction mixture was heated at 98°C for 30 seconds for denaturation, then cooled rapidly to 58°C with 2 min for annealing, and finally incubated at 72°C for 2 min for extension. These procedures were repeated 25 times. After the 1st PCR, 0.5 μl of solution was removed from each PCR mixture, and two PCR products were transferred to the mixture for the 2nd PCR consisting of ExTaq buffer, 0.2 mM dNTP mixture, and 3.0% DMSO in the total volume of 47.5 μl. The mixture was heated at 98°C for 10 min and cooled down to 37°C for 60 min with a linear gradinent. After incubation of 15 min at 37°C, 2.5 units of ExTaq DNA polymerase (0.5 μl) was added and the temperature kept at 72°C for 5 min. M13 primer M4 (20 pmol) and M13 primer RV (20 pmol) were added to the mixture (final volume: 50 μl), and the 2nd PCR was done under the same reaction conditions as those of the 1st PCR except for the annealing temperature (60°C). After the amplified fragments digested by *Eco*RI and *Hin*dIII were inserted into the *Eco*RI-*Hin*dIII sites of pUC118, DNA sequences of these fragments were analyzed to confirm the mutation introduced using ABI PRISM 310 Genetic Analyzer. Then, the amplified fragments were inserted into the *Eco*RI-*Hin*dIII sites of pKK223-3 for purification of mutant enzyme.

CD Measurements. CD spectra were measured at 25°C in 10 mM HEPES buffer, pH 7.0, with a Jasco spectropolarimeter model J-720. The CD spectra

were obtained at a protein concentration of 0.190–0.250 μg/ml in the far-UV region (200–250 nm) under a nitrogen atmosphere.

Metal analysis. The protein sample was analyzed with a Polarized Zeeman model Z-8100 atomic absorption spectrophotometer. The dialysis tube, plastic container, and water were free of metals.

Expression and purification of the wild-type and mutant enzymes. Previously, we reported the construction of high expression plasmids, pKNSD2, and purification of recombinant wild-type D-aminoacylase in *E. coli*.[24] All the mutant enzymes were purified fundamentally following the same procedure as that used for the recombinant wild-type enzyme, but for the purification of mutant enzymes, FPLC Mono Q HR 5/5 column (Pharmachia) was used with a 0 to 0.5 M NaCl linear gradient.

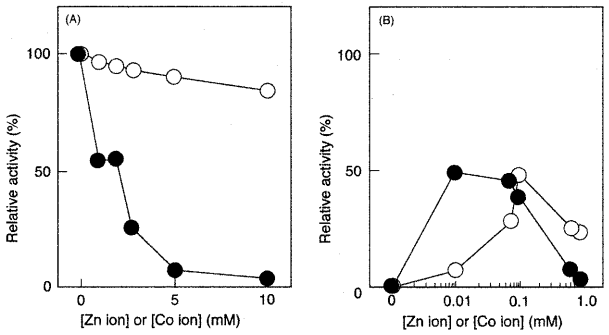

Fig. 1. Effects of Zn and Co ions on Native (A) and EDTA-treated (B) D-Aminoacylase Activity.

EDTA-treated D-aminoacylase was prepared by incubating the enzyme in 10 mM HEPES buffer (pH 7.0) containing 5 mM EDTA at 8°C for 20 min and then dialyzed against 10 mM HEPES buffer (pH 7.0). The enzyme activities of native or EDTA-treated enzyme were assayed under the standard assay conditions with various concentration of Zn ion (●) or Co ion (○). Relative activity was expressed using the activity of native enzyme as 100%.

Results

Effects of Zn and Co ions on enzyme activity

D-Aminoacylase from *Alcaligenes* A-6 treated with EDTA lost the activity, but about 50% activity was restored on addition of Zn (and Co) ion. An excess of Zn ion strongly inhibited the activity (Fig. 1). The activation/inhibition profile of zinc is similar to another D-aminoacylase from *Alcaligenes* DA181, which has zinc at the active center.[25]

Effects of site-specific reagents on enzyme activity

Enzyme activity was assayed under the standard conditions in the presence of 1 and 10 mM reagent (Table 2). The enzyme activity was especially strongly inhibited by DEPC. The incubation of enzyme with the product, D-leucine (10 mM) and competitive inhibitor, *N*-acetyl-D-alloisoleucine (10 mM) resulted in a loss of 58% and 75% of the enzyme activities by DEPC (1 mM), respectively, compared with the over 90% loss of activity observed in the absence of these compounds. A non-competitive inhibitor, glyoxylic acid, was not effective for the protection of the enzyme against the attack of DEPC.

Expression of mutant enzymes

Following the procedure described in Materials and Methods, the six plasmids responsible for the high production of His-mutants, H67N, H67I, H69N, H69I, H250N, and H251N, were constructed. Nucleotide substitutions at mutation sites were confirmed using DNA sequencing. *E. coli* JM109 cells were transformed with these expression plasmids. Among these six mutants, the four mutants, H67N, H67I, H250N, and H251N, were expressed successfully in soluble fractions of the cells as found by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). No

Table 2. Effects of Site-specific Reagents on Enzyme Activity

Reagents	Residual activity (%)	
	1 mM	10 mM
No addition	100	100
5′,5′-Dithiobis(2-nitrobenzoic acid)	83.5	58.5
N-Ethylmaleimide	90.3	72.8
Iodoacetate	117	63.3
N-Acetylimidazole	11.9	3.79
3-Bromopyruvate	98.6	72.4
Phenylglyoxal	44.9	1.67
Diethyl pyrocarbonate	7.10	0
4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride	83.3	57.2
Diisopropyl fluorophosphate	99.1	92.8
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide	84.1	54.6
2,4,6-Trinitrobenzeno-1-sulfonate	60.4	13.8

The enzyme was treated at 30°C for 20 min under the condition of pH specified in the use of the respective modifiers. The residual activity was assayed.

catalytic activity was detected in the homogenates from the transformants expressing the mutants H67N, H67I, H69N, and H69I. Little activity, below 1% of relative activity compared with wild-type enzyme, was detected in the homogenate corresponding to the H250N mutant. H251N mutant had approximately 30% of the activity of wild-type enzyme in the homogenate. Little enzyme protein was found in the soluble fractions of the mutants H69N and H69I. Debris of the mutants of both H69N and H69I after desruption of the cells was treated by 6 M urea for solubilization, and solubilized fractions were separated by SDS-PAGE. The protein highly expressed with the same molecular mass (about 52 kDa) as the wild-type enzyme was detected in each solubilized fraction of His69 mutants (data not shown). These results suggest that both H69N and H69I mutants formed inclusion bodies after expressed in the cells.

Purification and molecular properties of mutant enzymes

The wild-type and four mutant enzymes, H67N, H67I, H250N, and H251I, were purified to homogeneity judging from SDS-PAGE through DEAE-Toyopearl, Butyl-Cellulofine, and FPLC Mono Q column chromatography steps (data not shown). Because the activities of both H67N and H67I mutants were too low to measure in an inhomogenous state, the protein fractions corresponding to the mutant enzymes were recovered on the basis of the elution pattern of wild-type enzyme. But this procedure could not exclude the contamination completely. FPLC Mono Q column chromatography effectively excluded the contaminants from the mutant enzyme preparation after Butyl-cellulofine column chromatography. SDS-PAGE showed that each mutant comigrated with the wild-type enzyme to the position of 52 kDa (data not shown).

Kinetic properties of the mutant enzymes

The kinetic parameters, K_m and k_{cat}, for the wild-type D-aminoacylase and mutated enzymes are shown in Table 3. It was not possible to measure these constants for the inactive enzyme, H67I. H67N had a detectable D-aminoacylase activity, when compared to the totally inactive H67I. As the K_m for H67N mutant enzyme was not significantly different from that of wild-type enzyme, the substitution of His67 residue in this enzyme did not affect substrate binding. The k_{cat} for H67N mutant enzyme was, however, much lower than that of the wild-type enyzme, leading to decrease in k_{cat}/K_m of 6.3×10^4-fold. These results suggested that the His67 residue is essential for catalysis but not for substrate binding. The k_{cat} for H250N mutant enzyme strikingly decreased, and the K_m for this mutant increased approximately 6-fold of the wild-type enzyme. This suggests that the His250 residue might be involved in both catalysis and substrate binding. No significant difference of the K_m and k_{cat} between the H251N mutant enzyme and the wild-type enzyme could be found. An important role in catalysis could not be recognized in the His251 residue of D-aminoacylase.

Table 3. Kinetic Parameters of the Wild-type and Mutated Enzymes

Enzyme	K_m (mM)	k_{cat} (1/min)	k_{cat}/K_m (1/mM·min)
Wild-type	1.93	7.35×10^4	3.81×10^4
H67N mutant	1.44	0.88	0.61
H250N mutant	11.6	1.78×10	1.53
H251N mutant	0.61	3.56×10^4	5.83×10^4

CD spectra

CD spectra of the purified wild-type and four His-mutant enzymes were measured to see whether or not global conformational changes were brought about by the amino acid replacement. The spectra of mutant enzymes overlapped nearly with that of the wild-type enzyme in the wavelengths of 200–250 nm with molecular ellipticity (Fig. 2). This suggests that the replacements of His67, His250, and His251 with Asn or Ile do not significantly influence the secondary structure content of the enzymes, and therefore conformational changes caused by the mutations of these histidine residues are very minor.

Metal analysis

The purified wild-type D-aminoacylase contained about 2.3 g·atom of zinc per mole of enzyme. The contents of Ni and Ca ions were negligible. The mutant enzymes of H67N and H250N contained 2.2 and 0.7 g·atom of zinc per mole of enzyme, respectively.

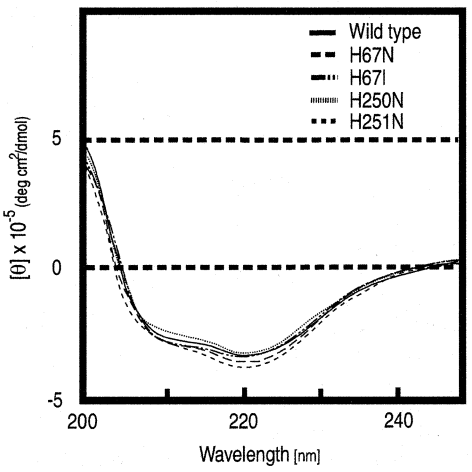

Fig. 2. Circular Dichroic Spectra of Wild-type and Mutant Enzymes.

CD spectra were taken in 10 mM potassium phosphate buffer (pH 7.0) at the enzyme concentration of 0.2 mg/ml.

Discussion

D-Aminoacylase from *Alcaligenes* A-6 was a Zn-enzyme judging from Fig. 1 and the Zn analysis. Chemical modification study with various site-specific reagents was done to clarify which amino acid residues are responsible for catalysis. Enzyme activity decreased drastically in the presence of 1 mM DEPC. However, incubation of the enzyme with a product, D-leucine, or a competitive inhibitor, *N*-acetyl-D-alloisoleucine, not completely, but partially reduced the inactivation by DEPC. A non-competitve inhibitor, glyoxylic acid, was not at all effective for the protection of the enzyme against the attack of DEPC. In *Pseudomonas* 5f-1, a histidine residue of D-AGase essential to catalysis could be protected by a substrate, *N*-acetyl-D-glutamate, and a competitive inhibitor, α-ketoglutaric acid, but not by a non-competitive inhibitor, glyoxylic acid.[23] These results also support the idea that histidine residues are important in hydrolytic catalysis at the active site. Therefore, histidine residues were selected as candidates for amino acid substitution to identify the amino acid residues responsible for catalysis. Asn and Ile were chosen as substituents for histidine because the characteristics of both side chains of Asn (uncharged polar) and Ile (nonpolar) are different from that of histidine (charged polar). His67, His69, His250, and His251 were changed to Asn or Ile by site-directed mutagenesis, and the kinetic properties, CD spectra, and Zn contents of resultant mutant enzymes were investigated. The significant roles of these histidine residues on both catalysis and structure were clarified. His69 mutant enzymes formed inclusion bodies, but other mutants were recovered in the soluble fractions. Comparison of CD spectra of His67, His250, and His251 mutant enzymes with wild-type enzyme indicated that no significant conformational change was caused by the mutations of these histidine residues. The k_{cat} of H67N mutant enzyme decreased drastically without a change of K_m. In the H250N mutant enzyme, a drastic decrease of the k_{cat} with a several-time increase of the K_m was observed. These results indicated that His67 and His250 are indispensable to catalysis.

His67 and His69, the targets for substitution of amino acid residues in this study, correspond to the His-X-His sequence, which is considered as a metal-binding motif of some of metal enzymes such as carbonic anhydrase,[26] β-lactamase,[27] and dihydroorotase,[28] D-AAase and D-AGase from *Alcaligenes* A-6 also have the same motif at almost the same position in their amino acid sequences. Atomic absorption spectra were measured to see whether or not the histidine residues, focussed in this study, are responsible for Zn binding. No significant difference of Zn contents between wild-type and mutant enzymes, except the H250N mutant, was observed. The Zn content of H250N mutant enzyme was only half of that of wild-type enzyme. Moreover, the H250N mutation largely decreased the enzyme activity. These results suggested that the His250 residue might be responsible for Zn binding and this Zn binding might be essential to catalysis, and that these was no involvement of the oxygen atom of Asn residue of H250N mutant enzyme in Zn-binding. These results also suggest that the His251 residue might not be responsible for both Zn-binding and catalysis, but that the His67 residue might be involved in either donating a hydrogen bond to the substrate or polarizing a water molecule, which subsequently attacks an amide bond. H69N and H69I mutant enzymes seem to have an unstable structure and His69 is a significant amino acid residue to conform to the proper tertiary structure and/or to maintain it. It has been reported that L-aminoacylase from porcine kidney requires Zn ion to maintain the catalytically active conformation at the active site,[29] and that the intrinsic Zn ion of thermostable L-aminoacylase from *B. stearothermophilus* has a the structural role.[30]

N-Acyl-D-amino acid amidohydrolases including D-aminoacylase from *Alcaligenes* A-6 have been reported to be all monomeric enzymes and require Zn ions for activity. Chai *et al.* reported that D-aminoacylase from *Alcaligenes denitrificans* DA181 has about 2.1 g·atom of zinc per mole of enzyme.[30] We showed that D-AGase from *Pseudomonas* sp. 5f-1 contains about 2.4 g·atom of zinc per mole of enzyme.[31] In this report, we showed that D-aminoacylase from *Alcaligenes* A-6 also contains about 2.3 g·atom of zinc per mole of enzyme. On the contrary, L-aminoacylase from porcine kidney is a homodimer, which contains one Zn ion per subunit.[32] Both enzymes from *Bacillus thermoglucosidius* and *B. stearothermophilus* have been reported to have about 1 g·atom of zinc per subunit.[29,33] All of them require Zn ions for catalytic reaction. Chemical modification studies for porcine kidney L-aminoacylase have suggested that at least four histidine residues participate in the binding of the essential two Zn ions per molecule, and that the enzymatic activity depends on the presence of at least two cysteine residues.[21,34] However, the histidine and cysteine residues involved in the active site of porcine kidney enzyme have not been specified. *N*-Acyl-D-amino acid amidohydrolases including D-aminoacylase and D-AGase exsist as a monomer containing two Zn ions and have the different structural characteristics from L-aminoacylase. D-Aminoacylase requires Zn ion to give the proper catalytic activity, but little information has been available concerning the amino acid residues involved in Zn binding as well as catalysis. L. Holm *et al.* described the striking

similarities of enzyme architecture including the active site between a urease from *Klebiella aerogenes* and the enzymes involved in nucleotide metabolism on the basis of three-dimensional structure of urease by X-ray crystallographic analysis while they had no relatives in the sequence database, and categorized these enzymes in a urease-related amidohydrolase superfamily. This superfamily includes dihydroorotase, allantoinase, hydantoinase, and atrazine-metabolizing enzymes etc.[35,36] The three closely related *N*-acyl-D-amino acid amidohydrolases (D-aminoacylase, D-AAase, and D-AGase) from *Alcaligenes* A-6 share significant sequence similarities with the members of the superfamily. The *N*-terminal H-X-H region also conserved in the three *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6 was proposed to contain metal-coordinating histidine residues in the members of this superfamily of enzymes. In D-aminoacylase from *Alcaligenes* A-6, His69 residue might participate in Zn binding. It has been shown that zinc endoprotease from *Streptomyces caespitosus*[37] and urease from *K. aerogenes*[38] use an aspartic acid residue for metal binding, Zn-binding and Ni-binding, respectively. In L-aminoacylase from porcine kidney, a cysteine residue has been proposed to be involved in Zn-binding.[39] Fourteen aspartic acid residues and one cysteine redidue are conserved among three *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6. In D-aminoacylase from *Alcaligenes* A-6, there is a possibility that some aspartic acid residues and one cysteine residue might also be involved in Zn-binding as well as the histidine residues. Further investigations are in progress to clarify the roles of these amino acid residues. Recently, it has been reported that D-aminoacylase from *Sebekia benihara*, which is not inhibited by EDTA, might be a metal-independent enzyme.[40] It is interesting to compare the structure-function relationships of metal-dependent D-aminoacylase with those of metal-independent enzyme.

In this report, we investigated the roles of histidine residues in the sequences, Asp-X_1-His^{67}-Thr-His^{69}-Asp-Asp and Ser-His^{250}-His^{251}-Lys conserved among three *N*-acyl-D-amino acid amidohydrolases from *Alcaligenes* A-6 by site-directed mutagenesis. The results suggested that His67 is essential for the catalytic event and that His69 is important to conform or to maintain the higher-order structure. The His250 residue might be responsible for Zn binding and this Zn binding might be essential to catalysis. At present, X-ray crystallographic studies of D-aminoacylase from *Alcaligenes* A-6 are under way to clarify the tertiary structure and the role of amino acid residues at the active site of this enzyme.

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