The Role of α-Amino Group of the N-terminal Serine of β Subunit for Enzyme Catalysis and Autoproteolytic Activation of Glutaryl 7-Aminocephalosporanic Acid Acylase*

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Glutaryl 7-aminocephalosporanic acid (GL-7-ACA) acylase of Pseudomonas sp. strain GK16 catalyzes the cleavage of the amide bond in the GL-7-ACA side chain to produce glutaric acid and 7-aminocephalosporanic acid (7-ACA). The active enzyme is an (αβ)_4 heterotetramer of two non-identical subunits that are cleaved autoproteolytically from an enzymatically inactive precursor polypeptide. In this study, we prepared and characterized a chemically modified enzyme, and also examined an effect of the modification on enzyme catalysis and autoproteolytic processing of the enzyme precursor. We found that treatment of the enzyme with cyanate ion led to a significant loss of the enzyme activity. Structural and functional analyses of the modified enzyme showed that carbamylation of the free α-amino group of the N-terminal Ser-199 of the β subunit resulted in the loss of the enzyme activity. The pH dependence of the kinetic parameters indicates that a single ionizing group is involved in enzyme catalysis with pK_a = 6.0, which could be attributed to the α-amino group of the N-terminal Ser-199. The carbamylation also inhibited the secondary processing of the enzyme precursor, suggesting a possible role of the α-amino group for the reaction. Mutagenesis of the invariant N-terminal residue Ser-199 confirmed the key function of its side chain hydroxyl group in both enzyme catalysis and autoproteolytic activation. Partial activity and correct processing of a mutant SI98T were in agreement with the general mechanism of N-terminal nucleophile hydrolases. Our results indicate that GL-7-ACA acylase utilizes as a nucleophile Ser-199 in both enzyme activity and autoproteolytic processing and most importantly its own α-amino group of the Ser-199 as a general base catalyst for the activation of the hydroxyl group both in enzyme catalysis and in the secondary cleavage of the enzyme precursor. All of the data also imply that GL-7-ACA acylase is a member of a novel class of N-terminal nucleophile hydrolases that have a single catalytic center for enzyme catalysis.

The glutaryl 7-aminocephalosporanic acid (GL-7-ACA)¹ acylase (EC 3.5.1.11) of Pseudomonas sp. strain GK16 catalyzes the hydrolysis of GL-7-ACA to release glutaric acid and 7-aminocephalosporanic acid (7-ACA) (1, 2) (Fig. 1). The active enzyme is a heterotetramer consisting of two α and two β subunits (2, 3). Cephalosporin acylases including this enzyme have long been of industrial interest in the synthesis of semi-synthetic cephalosporin antibiotics but their catalytic mechanisms are still poorly understood. The nascent polypeptide of the enzyme is synthesized as a 74-kDa polypeptide containing a signal peptide at its N terminus. After the removal of the signal peptide, an enzymatically inactive 70-kDa precursor polypeptide is activated by proteolytic cleavages into two subunits, 16-kDa α and 54-kDa β subunits, in the periplasm (3, 4). In a previous study (4), we have proposed that the enzyme is activated through a two-step autoproteolytic processing upon folding: the first step is an intramolecular cleavage of the precursor between Gly-198 and Ser-199 for generation of the inactive α subunit containing a spacer peptide of 9 amino acids and the β subunit; the second is an intermolecular event, which may be mediated by the N-terminal Ser (Ser-199) of the β subunit and results in a further cleavage of the peptide bond between Gly-189 and Asp-190 and the removal of the spacer peptide. Our previous data have also shown that Ser-199, the N-terminal residue of the β subunit, is essential for the catalytic activity and autoproteolytic activation of the enzyme (4).

N-terminal nucleophile (Ntn) hydrolases form a novel class of hydrolytic enzymes. They are activated from an enzymatically inactive precursor polypeptide by proteolytic processing, producing a new N-terminal residue. Several amidohydrolases belonging to the Ntn hydrolase family have been described with very different substrate specificities and functions (5). Glutaminyl 5-phosphoribosyl-1-pyrophosphate amidotransferase (6), penicillin acylase (7), proteasome β subunit (8), and aspartyl-glucosaminidase (9) all have a similar central four-layer sandwich of α helices and β sheets (αββα) as a catalytic domain and an N-terminal nucleophile responsible for catalyzing the hydrolysis of an amide bond.

The N-terminal residue, which is exposed upon the proteolytic cleavage of the precursor polypeptide, has been shown to be essential for the enzymatic activity in the Ntn hydrolases. This residue is threonine in aspartylglucosaminidase and proteasome β subunit, serine in penicillin acylase, and cysteine in glutaminyl 5-phosphoribosyl-1-pyrophosphate amidotransferase. All of these residues can function as a catalytic nucleophile and are located at the beginning of a β strand. In the case of aspartylglucosaminidase and penicillin acylase activation, the precursor polypeptide chain is cleaved into two polypeptide subunits of the active protein, whereas in proteasome

nucleophile; Bis-Tris, 2-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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¹ The abbreviations used are: GL-7-ACA, glutaryl 7-aminocephalosporanic acid; 7-ACA, 7-aminocephalosporanic acid; Ntn, N-terminal
and glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase, the activation results in the removal of a propeptide. Recently, a number of studies have suggested that the breaking of this critical peptide bond occurs autocatalytically and that the autoprocessing is initiated by a proximal N-O or N-S acyl shift which occurs by nucleophilic attack by Ser, Cys, or Thr (10–16). The suggested catalytic mechanisms of these four enzymes contain another interesting feature in common that has only been described for them. It has been suggested that the α-amino group of the N-terminal catalytic amino acid could function as a base that increases the nucleophilicity of the hydroxylthiol group (5, 7, 9, 17).

Here, we report the first experimental study that probes the function of the free α-amino group of the N-terminal catalytic amino acid within the family of Ntn hydrolases. Our results suggest that the α-amino group of GL-7-ACA acylase is an essential catalytic group, acting as a general base catalyst by deprotonating the hydroxyl group of the N-terminal Ser-199 of the β subunit in enzyme catalysis and possibly in secondary processing of the enzyme precursor. Mutagenesis of the invariant N-terminal Ser-199 confirmed the key function of its side chain hydroxyl group as a putative nucleophile in both enzyme catalysis and autoproteolytic activation. Our data would also imply that GL-7-ACA acylase is a member of a novel family of structurally similar hydrolytic enzymes with an analogous catalytic mechanism resembling that of serine proteases and in which the N-terminal amino acid of a polypeptide chain acts both as the nucleophile and the base.

**EXPERIMENTAL PROCEDURES**

**Materials**—The sources of materials used were as follows: potassium cyanate was from Fluka; Bio-Gel P-6 DG desalting gel from Bio-Rad; Sephacel C18 column from Amersham Pharmacia Biotech; Lys-C endopeptidase of sequencing grade from Roche Molecular Biochemicals; GL-7-ACA from ChongKunDang Co. All other chemicals were of analytical grade or the highest quality commercially available.

**Protein Purification**—GL-7-ACA acylase with the C-terminal (His)_6 tag was purified using Talon™ metal-charged affinity columns as described previously (4).

**Chemical Modification of GL-7-ACA Acylase with KCNO**—Purified GL-7-ACA acylase (12 μg) was titrated with increasing concentrations of KCNO (10–160 mM) in 20 mM Tris-HCl, pH 8.0. The reaction mixtures were incubated at 37 °C for 1 h. After incubation, each of the mixtures was gel filtered on Bio-Gel P-6 DG spin columns to terminate the modification reaction. The residual GL-7-ACA acylase activity was measured as described later.

**Purification and Lys-C Digestion of GL-7-ACA Acylase**—GL-7-ACA acylase (600 μg) was incubated with 1 μM KCNO in 500 μL of 20 mM Tris-HCl, pH 8.0, at 37 °C for 2 h. This treatment resulted in a 95% reduction of the enzyme activity. Modified and unmodified enzymes were separately dialyzed against 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 6 mM guanidine HCl at 4 °C. The β subunits from modified and unmodified

**enzymes were purified as described previously (4), and then dialyzed extensively against 25 mM Tris-HCl, pH 8.0. Each of the purified β subunits (77 μg) was digested with the protease Lys-C in an enzyme to substrate ratio of 1:100 (w/w) at 37 °C for 24 h. The reaction mixtures were then applied to a Sephacel C18 reverse-phase column (12.1 × 160 mm) in SMART system (Amersham Pharmacia Biotech) and eluted at a flow rate of 0.2 ml/min with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The column eluate was monitored at 214 nm.

**Mass Spectrometry and Amino-terminal Sequence Analysis**—Each of the entire Lys-C digests of modified and unmodified β subunits was desalted using ZipTip™ (Millipore), and then mixed together with an equal volume of α-cyano-4-hydroxycinnamic acid solution. The sample/matrix solution was dropped onto a sample plate for matrix-assisted laser desorption ionization time of flight mass spectrometry, and then dried under ambient conditions. A mass spectrum was obtained on a Kratos Kompact MALDI II instrument (Kratos). N-terminal amino acid sequence analysis was performed on an Applied Biosystems 491 protein sequencer fitted with a high-pressure liquid chromatography on-line system.

**Site-directed Mutagenesis**—Point mutations to form mutants S199T and K208Q were introduced into pSH, a plasmid that contains a sequence coding for mature wild-type GL-7-ACA acylase with a C-terminal (His)_6 tag downstream from a T7 RNA polymerase promoter site (4), using overlap extension polymerase chain reaction method (18). All the mutations were verified by automated DNA sequencing to ensure that only the desired mutation and no other changes were inserted into the sequence.

**GL-7-ACA Acylase Activity Assay**—The assay for GL-7-ACA acylase activity was based on the colorimetric measurement of 7-ACA released from the substrate GL-7-ACA (4). For pH profiles of enzyme activity, purified wild-type and S199T mutant enzymes were diluted into the buffers with different pH values. For pH 4–6, 20 mM citric acid/Na_2HPO_4 buffers were used. For pH 7–8 and 9–10, 20 mM potassium phosphate and 20 mM bis-Tris-propane/HCl buffers were used, respectively. Determination of the K_m and V_max values for wild-type and S199T mutant enzymes was performed at varying substrate concentrations at pH 7.0.

**pH Dependence of Kinetic Parameters**—The following pH range was explored: citric acid/Na_2HPO_4, pH 5–6.5, sodium phosphate, pH 7–8, bis-Tris-propane/HCl, pH 8.5–9.5. All the buffers were 20 mM, and the ionic strength of the solution was kept at 0.1 M by adding NaCl. The kinetic parameters were obtained by determining initial rates at various concentrations of the substrate at different pH values. The apparent pH value governing the pH dependence of steady-state kinetic parameters was extracted from y versus pH profiles fitted to the following general equation,

\[
y = y_{\text{lim}}(1 + 10^{pK_a - pH})
\]

where y_{lim} represents the maximum pH-independent value.

**RESULTS**

**Effect of KCNO Treatment of GL-7-ACA Acylase on Enzyme Activity**—The recently described Ntn hydrolases suggest that the α-amino group of the N-terminal catalytic amino acid of the enzymes may play an essential role as a general base to enhance the nucleophilicity of the hydroxylthiol group (5, 7, 9, 17). As the penicillin acylase of these enzymes is functionally homologous to GL-7-ACA acylase, the α-amino group of the N-terminal Ser-199 of the β subunit of GL-7-ACA acylase may be critical for the enzyme activity. To examine this possibility, we attempted to carbamylate the α-amino group of the N-terminal Ser-199 by treating the enzyme with KCNO under various conditions, and the chemically modified derivatives of the enzyme were examined for their enzyme activity. As shown in Fig. 2, the incubation of GL-7-ACA acylase with KCNO led to a progressive loss of enzyme activity in a concentration-dependent manner, suggesting the importance of one or more amino group-containing residues.

**Determination of the Site of Modification Responsible for the Loss of Enzyme Activity of GL-7-ACA Acylase**—We first examined which carbamylated subunit of GL-7-ACA acylase was responsible for the loss of enzyme activity. Since enzyme activity can be regained by enzyme reconstitution with inactive α
and β subunits (4), the α and β subunits were purified from unmodified and modified GL-7-ACA acylases, and mixed in various combinations for enzyme reconstitution. Table I shows that the modification in the β subunit results in enzyme inactivation.

To identify the carbamylated residue, we used β subunits purified from intact and carbamylated GL-7-ACA acylases. The β subunits were subjected to Lys-C endopeptidase digestion, after which the Lys-C digests were separated by reverse-phase high performance liquid chromatography. The differences observed between the two elution profiles were a single shift of an arrow-labeled peak which only appears as a minor shoulder in the HPLC chromatogram of carbamylated sample (Fig. 3, A and B). Because the peak of overlapping peptides including the carbamylated peptide seen in Fig. 3B was not further separated by means of a shallow water/acetonitrile gradient, we analyzed the entire Lys-C digests of intact and carbamylated β subunits by matrix-assisted laser desorption ionization time of flight. The mass spectrometric analyses clearly allowed us to find a new peptide having an additional 43 mass units, not found among peptides from the Lys-C digest of intact β subunit (Table II). The single difference of molecular masses between the Lys-C digests of intact and carbamylated β subunits corresponds to the mass of a carbamyl adduct, suggesting that the peptide having an additional 43 mass units is a peptide corresponding to residues from N-terminal Ser-199 of the β subunit (Table II). The N-terminal sequence analysis on the arrow-labeled peak that appears from uncaramylated sample. For the N-terminal sequence analysis of carbamylated β subunit itself, no phenylthiohydantoin-derivative of amino acid was detected. Also, an active R208Q mutant was still sensitive to enzyme inactivation with KCNO, and treatment of wild-type enzyme with 1,3,5-trinitrobenzenesulfonate, a specific lysine modifier, had no effect on enzyme activity (data not shown). All the results indicate that the single site modification of an α-amino group of the N-terminal Ser-199 occurred on the β subunit and led to the inactivation of GL-7-ACA acylase.

Effect of the Carbamylated N-terminal Ser of β Subunit on the Secondary Processing of GL-7-ACA Acylase Precursor—By enzyme reconstitution experiments, we have previously revealed that the GL-7-ACA acylase precursor undergoes the secondary processing catalyzed by the N-terminal Ser-199 of the β subunit, which is generated from the primary processing of the precursor (4). To examine whether the carbamylation of the N-terminal Ser-199 of the β subunit affects the secondary processing, each of the intact and carbamylated β subunits was mixed and reconstituted with α subunit containing a spacer peptide from a S199C mutant, which undergoes only the primary processing of its precursor (4). As shown in Fig. 4, the carbamylated N-terminal Ser of the β subunit was not able to catalyze the secondary processing, i.e. the removal of the spacer peptide, suggesting that the α-amino group of the N-terminal Ser-199 of the β subunit also plays an important role in the secondary processing of the precursor as well as enzyme catalysis.

Effects of a S199T Mutation on Enzyme Activity and Autoproteolytic Activation of GL-7-ACA Acylase—Previous substitutions of the β-subunit N-terminal Ser-199 by Cys or Ala suggested that the N-terminal Ser is essential both for enzyme catalysis and for complete processing of the precursor (4). To investigate whether a hydroxyl-containing residue at the position is critical for the autoproteolytic activation and enzyme activity of GL-7-ACA acylase, Ser-199 was replaced with Thr using site-directed mutagenesis. The conservative replacement of Ser-199 with Thr had no effect on the correct cleavage of the enzyme precursor into α and β subunits but greatly affected the enzyme activity, which was reduced to only 13% that of wild

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**Table I**

| Components | Enzyme activity |
|------------|----------------|
| α (-) + β (-) | 100 |
| α (-) + β (+) | 8.2 |
| α (+) + β (-) | 100 |
| α (+) + β (+) | 7.6 |
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The elution medium.

of GL-7-ACA acylase.

0.1% trifluoroacetic acid. The column eluate was monitored at 214 nm flow rate of 0.2 ml/min with a linear gradient of acetonitrile containing a Sephacel C18 reverse-phase column (2.1 kDa in this paper as mentioned by Matsuda and Komatsu (3).

N-terminal Ser of the subunit.

zyme, both kcat and km values of the S199T mutant were reduced about 25-fold, but kcat/km values were almost the same for both enzymes (Table III).

We also measured the relative enzyme activity of wild-type and S199T mutant enzymes at different pH values to detect variations in the pH optimum (Fig. 5). The S199T mutant enzyme showed some alterations in its pH optimum profiles. The activity of the wild type increased as the pH rose, having a broad optimum range between pH 6 and 10, while the S199T mutant had a relatively narrow range of pH optimum from pH 6 to 8, after which the activity declined sharply. However, it was questionable whether the decline in enzyme activity of S199T mutant at pH > 8.0 is due to an irreversible inactivation of the enzyme. To answer this issue, the enzyme was preincubated at various pH values in the range of 5–10 for the usual assay time, and the activity was then measured after buffer exchange into pH 7.0 buffer by spin column chromatography. Whereas the enzyme preincubated between pH 5 and 8 regained full activity at pH 7.0, irreversible inactivation for the enzyme increased rapidly above pH 8.0 (data not shown), indicating that the decline of the enzyme activity in the pH profile of S199T mutant enzyme must result from the irreversible inactivation of the enzyme.

pH Dependence of the Kinetic Parameters—If the α-amino group of the N-terminal Ser-199 of the β subunit of GL-7-ACA acylase is responsible for deprotonating the hydroxyl group of Ser-199, then the enzyme would be active only when this acidic group is unprotonated in the enzyme. The pH dependence of the reaction rate should indicate the presence of an ionizable group that must be unprotonated for enzyme catalysis. Thus, we generated pH profiles of the steady-state kinetic parameters, kcat and km/kcat, of the reconstituted enzyme mixtures. STD and pro stand for protein standards and the spacer peptide, respectively.

(type. In combination with the inability of Cys to replace the N-terminal Ser of the β subunit for the enzyme activity and secondary processing of the precursor, these results indicate that Ser-199 is critical not only for two steps of processing but also for catalysis as a nucleophile. Relative to wild-type enzyme, both km and kcat values of the S199T mutant were
of both $k_{cat}/K_m$ and $k_{cat}$ revealed a critical ionization that must be unprotonated for catalysis (Fig. 6). As the pH increases, there is a corresponding increase in catalytic activity that plateaus above the critical ionization with $pK_a$ values of $5.6 \pm 0.1$ for $k_{cat}$ and $6.0 \pm 0.1$ for $k_{cat}/K_m$. The S199T mutant showed similar results of $pK_a$ values of $5.7 \pm 0.1$ for $k_{cat}$ and $6.0 \pm 0.1$ for $k_{cat}/K_m$. These data indicate the catalytic requirement of an unprotonated active site group in the enzyme. The slight difference of $pK_a$ values for $k_{cat}$ and $k_{cat}/K_m$ also imply little change in the states of ionization of the catalytic center group on formation of the acyl-enzyme.

**DISCUSSION**

To explore the possibility of general base catalysis by the free $\alpha$-amino group of the N-terminal Ser-199 of the $\beta$ subunit of GL-7-ACA acylase, we treated the enzyme with cyanate ions under controlled conditions, and identified a single amino group essential for the catalytic activity of the enzyme. We also examined the pH dependence of the kinetic parameters of wild-type and S199T mutant enzymes. We found that carbamylation of the $\alpha$-amino group of the N-terminal Ser-199 of GL-7-ACA acylase led to a significant loss of its enzyme activity, indicating that the $\alpha$-amino group is essential for the catalytic activity of the enzyme. Penicillin acylase (7), aspartylglucosaminidase (9), proteasome $\beta$ subunit (8), and glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase (6) have been described and suggested to form a novel class of Ntn hydrolases (5). All these hydrolases have N-terminal catalytic amino acids of Ser, Thr, or Cys. The suggested mechanisms of these enzymes for catalysis contain an interesting feature in common that has only been described for them. Because no adjacent histidine equivalent to that found in the serine proteases is in close enough proximity to the N-terminal catalytic amino acid in the crystal structures of Ntn hydrolases, the proposal was made that the $\alpha$-amino group of the N-terminal catalytic amino acid functions as a base that increases the nucleophilicity of the hydroxy/thiol group. The $\alpha$-amino group of the N-terminal catalytic amino acid is suitably positioned to mediate proton transfers directly or via a bridging water molecule (5, 7, 9, 17). If the $\alpha$-amino group of GL-7-ACA acylase has a same role for enzyme catalysis as those of the Ntn hydrolases, it is speculated that the carbamylation of the N-terminal Ser-199 of GL-7-ACA acylase must lead to a reduction of basicity of the $\alpha$ nitrogen of the $\alpha$-amino group, which probably makes it unable to act as a general base to enhance the nucleophilicity of the hydroxyl group, thereby abolishing the enzymatic activity.

An active site base is required to catalyze proton transfers in overall catalysis, and we thus determined the pH dependence of $k_{cat}$ and $k_{cat}/K_m$ of wild-type and S199T mutant enzymes. The $k_{cat}/K_m$ pH profile of the wild type indicates that a single ionizable group with a $pK_a$ of $6.0 \pm 0.1$ has to be unprotonated for activity. The $k_{cat}$ pH profile is very similar, except that the $pK_a$ is shifted to $5.6 \pm 0.1$. It is likely that the acidic group revealed in the $k_{cat}/K_m$ and $k_{cat}$ pH profiles corresponds to the same ionizable group. Typically, the $k_{cat}/K_m$ pH profile represents $pK_a$ values of residues in the free enzyme or free substrate and the $k_{cat}$ pH profile represents $pK_a$ values in the enzyme-substrate complex (19). The $pK_a$ value from the $k_{cat}/K_m$ pH profile most likely represents an ionizable group in free enzyme, since the free substrate does not have an ionizable group in the pH range tested. The $pK_a$ values from the $k_{cat}/K_m$ and $k_{cat}$ pH profiles of the S199T mutant enzyme were similar to those of the wild type, suggesting that the same ionizable group as the wild type is required for the catalytic activity of the S199T mutant. Therefore, the pH dependence of the kinetic parameters in GL-7-ACA acylase indicates that a group with an apparent $pK_a$ of 6.0 in the free enzyme is critical for activity. Although the value of apparent $pK_a$ is somewhat less than a $pK_a$ value of 6.8–7.9 expected for an $\alpha$-amino group, a decreased value is plausible in a protein environment that is less polar than that of the aqueous solvent. A similar result is seen in penicillin acylase, a functional homologue of GL-7-ACA acylase and a member of the Ntn hydrolase family, in which the $pK_a$ value of the free $\alpha$-amino group of the N-terminal catalytic Ser was assigned to 6.1 (20). Since histidine’s first $pK_a$ value is 6.2, however, we could not exclude a possibility that a His residue may function as a general base in the enzyme catalysis. Based on a multiple amino acid sequence alignment of cephalosporin acylases and a comparative modeling study with the

**TABLE III**

| Protein     | $K_M$ | $k_{cat}$ | $k_{cat}/K_M$ |
|-------------|-------|-----------|--------------|
|             |       | $s^{-1}$  | $s^{-1}m^{-1}$|
| Wild type   | 1.05  | 9.48      | 9.03         |
| S199T       | 0.04  | 0.35      | 8.75         |

*Assays of purified wild-type and S199T mutant GL-7-ACA acylases.*

*Fig. 5. The pH optimum profiles of wild-type and S199T mutant GL-7-ACA acylases.* The enzyme activity was measured with wild-type and S199T mutant enzymes at various pH values as described under “Experimental Procedures.” Results are shown for wild type (open circles) and S199T mutant (closed circles).
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Ser-199.

In this study, although it could not be clearly explained why the S199T mutant has reduced $K_m$ value and is unstable at high pH as compared with the wild type, Thr did offer somewhat an acceptable replacement for the enzyme activity. This suggests that either hydroxyl group of serine or threonine can function as a nucleophile in the enzyme catalysis. This result is consistent with the general mechanism of Ntn hydrolases in which either Ser, Cys, or Thr functions as the nucleophile.

Carbamylation of the N-terminal Ser-199 of the β subunit completely inhibited the secondary processing (Fig. 4), which is catalyzed by the N-terminal Ser-199 generated from the primary processing of the enzyme precursor and results in the removal of a spacer peptide (4). Moreover, in vitro processing of the GL-7-ACA acylase precursor is maximal between pH 7.0 and 10.0 (data not shown). It is noteworthy that this behavior is similar to the pH-dependent profile for GL-7-ACA acylase activity. Accordingly, the inhibition of the secondary processing by carbamylation could be explained by speculation that carbamylation of the α-amino group of the N-terminal Ser makes it unable to act as a general base to activate the hydroxyl group of the N-terminal Ser, since precursor and mature enzyme may share a similar structure (16). In addition, the replacement of Ser-199 with Thr did not affect the correct processing into α and β subunits, suggesting that Thr could offer an acceptable replacement for autocatalytic processing of the enzyme precursor. Overall, these results suggest that a hydroxyl side chain (Ser or Thr) at position 199 is required for the complete processing reaction of the GL-7-ACA acylase precursor. According to the data, the side chain of Ser-199 would play an active role as a nucleophile in the autoproteolytic activation of GL-7-ACA acylase.

The present data allow us to conclude that the single N-terminal amino acid (Ser-199) of GL-7-ACA acylase can function as a nucleophile and a base in a way similar to that of serine and histidine residues in the active sites of serine proteases. Also, our data suggest that GL-7-ACA acylase utilizes Ser-199 as a nucleophile for both the primary and secondary processing and that the free α-amino group exposed from the primary processing could act as a general base for the secondary processing reaction. Taking all available experimental data together, we propose that GL-7-ACA acylase from *Pseudomonas* sp. strain GKI6 is a member of a recently described novel class of Ntn hydrolases. X-ray three-dimensional structure analysis on the GL-7-ACA acylase that is underway by us (22) will be able to reveal the detailed catalytic mechanism of the enzyme and to encourage the studies on the catalytic and autoproteolytic activation mechanisms of the enzyme. Further work is required to characterize and understand all biochemical aspects of GL-7-ACA acylase, from autoproteolytic activation to catalysis.

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REFERENCES

1. Ichikawa, S., Murai, Y., Yamamoto, S., Shibuya, Y., Fujii, T., Komatsu, K., and Kodaïra, R. (1981) *Agric. Biol. Chem.* 45, 2225–2229

2. Ichikawa, S., Shibuya, Y., Matsumoto, K., Fujii, T., Komatsu, K., and Kodaïra, R. (1981) *Agric. Biol. Chem.* 45, 2231–2236

3. Matsuda, A., and Komatsu, K. (1985) *J. Bacterial.* 163, 1222–1228

4. Lee, Y. S., and Park, S. S. (1998) *J. Bacterial.* 180, 4576–4582

5. Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C., Smith, J. L., Tomchick, D. R., and Murzin, A. G. (1995) *Nature* 378, 416–419

6. Smith, J. L., Zaluzec, E. J., Wery, J. P., Niu, L., Switzer, R. L., Zalkin, H., and Silbatow, Y. (1994) *Science* 264, 1427–1433

7. Duggleby, H. J., Tolley, S. P., Hill, C. F., Dodson, E. J., Dodson, G., and Moody, P. C. E. (1995) *Nature* 374, 264–268

8. Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995) *Science* 268, 535–539

9. Ponsen, C., Tikkanen, R., Rouvinen, J., and Peltonen, L. (1995) *Nat. Struct.*
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10. Guan, C., Cui, T., Rao, V., Liao, W., Benner, J., Lin, C.-L., and Comb, D. (1996) J. Biol. Chem. 271, 1732–1737
11. Schmidtke, G., Kraft, R., Kostka, S., Henklein, P., Frommel, C., Löwe, J., Huber, R., Kloetzl, P. M., and Schmidt, M. (1996) EMBO J. 15, 6887–6898
12. Seemüller, E., Lupas, A., and Baumeister, W. (1996) Nature 382, 468–471
13. Tikkanen, R., Rikonen, A., Oinonen, C., Rouvinen, R., and Peltonen, L. (1996) EMBO J. 15, 2894–2960
14. Guan, C., Liu, Y., Shao, Y., Cui, T., Liao, W., Ewel, A., Whitaker, R., and Paulus, H. (1998) J. Biol. Chem. 273, 9695–9702
15. Saarelä, J., Laine, M., Tikkanen, R., Oinonen, C., Jalanko, A., Rouvinen, J., and Peltonen, L. (1998) J. Biol. Chem. 273, 25320–25328
16. Xu, Q., Buckley, D., Guan, C., and Guo, H.-C. (1999) Cell 98, 651–661
17. Seemüller, E., Lupas, A., Stock, D., Löwe, J., Huber, R., and Baumeister, W. (1995) Science 268, 579–582
18. Hayashi, N., Welschof, M., Zewe, M., Braunagel, M., Dubel, S., Breitling, F., and Little, M. (1994) BioTechniques 17, 314–315
19. Cleland, W. W. (1982) Methods Enzymol. 87, 309–405
20. Morillas, M., Goble, M. L., and Virden, R. (1999) Biochem. J. 338, 233–239
21. Tanford, C. (1962) Adv. Protein Chem. 17, 69–165
22. Kwon, T. H., Rhee, S., Lee, Y. S., Park, S. S., and Kim, K. H. (2000) J. Struct. Biol. 131, 79–81