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Identification of a New Active Site for Autocatalytic Processing of Penicillin Acylase Precursor in *Escherichia coli* ATCC11105

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Penicillin acylase (PA) from *Escherichia coli* ATCC11105 is a periplasmic heterodimer consisting of a 24 kDa small subunit and a 65 kDa large subunit. It is synthesized as a single 96 kDa precursor and then matures to functional PA via a posttranslational processing pathway. The GST-PA fusion protein expression system was established for monitoring the precursor PA processing *in vitro.* The purified PA precursor was processed into mature PA the same way as *in vivo,* but pH dependently. From the primary sequence analysis, we identified a putative conserved lysine residue (K_{299}) responsible for the pH dependent processing. The substitution of K_{299} residue by site-directed mutagenesis affected both the enzyme activity and the precursor PA processing *in vivo.* Furthermore, it was shown that the processing rates of wild-type and mutant precursor PAs depended on the pKa values of their side chain R group. These results demonstrated that the lysine residue (K_{299}) was involved in the precursor processing of PA together with N-terminal serine residue (S_{290}) of the large subunit.

Key Words: **lysine; site-directed mutagenesis; *in vitro* processing; autocatalytic processing; penicillin acylase; *E. coli.***

Penicillin acylase (PA) hydrolyzes benzylpenicillin to give 6-amino penicillanic acid (6-APA), an intermediate in the production of semisynthetic penicillin (1). It consists of two nonidentical large and small subunits held together by noncovalent forces (2). PA of *E. coli* is encoded by a single gene and is initially synthesized as a single polypeptide that is processed posttranslationally. Several different proteolytic cleavages are necessary to generate the functional enzyme. The first cleavage removes a 26 amino acid signal peptide from the precursor protein. At least four subsequent proteolytic cleavages are required to remove the 54 amino acid spacer peptide, thereby producing its catalytically active form in the periplasm (3–5). For the active enzyme, the cleavage of the peptide bond between T_{289} and S_{290} is essential. After this cleavage, the spacer peptide is processed out through at least three steps (2).

In enzyme catalysis, PA is a member of the recently recognized structural superfamily of enzymes termed N-terminal nucleophile (Ntn) amidohydrolyases (6–10). In Ntn hydrolyases, N-terminal amino acid plays a critical role in the enzyme catalysis not only as a reaction nucleophile but also as a base to enhance the nucleophilicity. PA also has an N-terminal serine residue (S_{290}) that act not only as a nucleophile but also as a base in enzyme catalysis (3, 11).

In contrast with enzyme catalysis, the mechanism of precursor processing of PA is not well known. It was previously reported that the substitution of S_{290} of PA with Cys had inactivated the enzyme activity but resulted in normal processing (2). This implies that the mechanism of PA processing differs from that of enzyme catalysis. Recently, it was reported that PA from *E. coli* and GL 7-ACA from *Pseudomonas* were activated by intramolecular autoproteolysis (12, 13). These results suggest that precursor PA has an intrinsic active site for autocatalytic processing that differs from mature PA.

In the present study, we established a GST-precursor PA fusion protein expression system, and then investigated their processing patterns *in vitro.* We show that the *in vitro* processing of precursor PA exhibits the same pattern as *in vivo,* and also pH dependent activation. We find by amino acid sequence alignment that a conserved lysine residue (K_{299}), which is closely located to the N-terminal serine residue (S_{290}), could be the most probable candidate responsible for the pH dependent activation. The mutation of K_{299} affected the precursor processing and enzyme activity

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of PA *in vivo* and *in vitro*. Also, Arg and His substituents show a pH dependent processing pattern. Taken together, our results suggest a possible active center that includes both S_{290} and K_{299} in the activation of precursor PA processing.

MATERIALS AND METHODS

Bacterial strain, media, and growth condition. The bacterial strain used in this study was *E. coli* $DH5_{\alpha}$ ($supE44_{\Delta}lacU169$ ($\varnothing 80lacZ_{\Delta}M15$) hsdR17 recA1 endA1 gyrA96 thi-1 relA1). *E. coli* strains with plasmid were grown in Luria broth at 28°C except for the fusion protein purification. When required, the appropriate antibiotics were added at the following concentrations: ampicillin (50 μg/ml), kanamycin (50 μg/ml) and chloram-phenicol (34 μg/ml). Plasmid constructions and restriction enzyme analysis were carried out as described by Maniatis *et al.* (14).

Site-directed mutagenesis. Mutants in penicillin acylase gene (*pga*) were obtained by polymerase chain reaction using the overlap extension technique described by Ho *et al.* (15). For each mutation, a set of two overlapping primers (primer B; 5′-CGCGGGGTACC-CAACGACC-3′, and Primer H; 5′-TCCTGGGCATGGCTTTTGCCG-3′, Q; 5′-TCCTGGGCTTGGCTTTTGCCG-3′, R; 5′-TCCTGGGC-TCTGCTTTTGCCG-3′, S; 5′-TCCTGGGCTGAGCTTTTGCCG-3′) were used in combination with the primer A; 5′-CTCGCAAAC-TGCAGCTCTG-3′ and primer C; 5′-GACGACTGGTTTGGCGAA-3′. The mutagenized fragments were digested with *BstXI* and *BgIII* and replaced with the 0.8 kb *BstXI-BgIII* region in plasmid pUPD182, in which wild-type *pga* gene with upstream 182 bp had been cloned in plasmid pBR322. The resulting plasmids were named pK299H, pK299Q, pK299R and pK299S. The substituted regions were confirmed by DNA dideoxy sequence analysis.

Construction of a GST-PA fusion protein expression system. The plasmid pGEX-2T (Pharmacia) was used for construction of the pGT-AATS, which encode wild-type *pga* fused with GST gene. The DNA fragment containing part of the precursor PA coding region was prepared by *NcoI* partial digestion of plasmid pJA (2). The ends of this fragment were blunted and then ligated with pGEX-2T that had been digested with *BamHI* and treated with Klenow enzyme. DNA sequencing around the fusion point confirmed that the plasmid had a correct open reading frame. To express the mutant gene for GST-PA fusion proteins at lysine residue (K_{299}), the 1.5 kb *SphI-BgIII* DNA fragment containing the mutation was replaced with that of plasmids pK299H and pK299R.

Expression and purification of GST-PA fusion protein. The fusion proteins were purified from *E. coli* $DH5_{\alpha}$ in a single step with glutathione sepharose-4B (Pharmacia) beads as recommended by the manufacturer. The expression of fusion protein was performed at 23°C in L broth. Cells were grown to A_{600} of 0.5 and IPTG was added to the culture medium to a final concentration of 0.1 mM to induce *pga* gene expression. After 2 h induction, the cells were harvested by centrifugation (5000*g*, 5 min, 4°C). Harvested cells were washed once with ice-cold NET buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA). Cells were then suspended in the same buffer and broken by mild sonication. The disrupted cells were centrifuged at 10,000*g* for 15 min. The supernatant was mixed at 4°C with 1 ml of 50% glutathione sepharose-4B pre-equilibrated with NET buffer in a 15 ml tube on a rotary shaker. After adsorption for 2 h, beads were collected by brief centrifugation at 500*g*. Fusion proteins bound to beads were washed five times with 10 volumes of NET buffer and twice with thrombin cleavage buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 2.5 mM $CaCl_2$). Thrombin protease was added to the beads to a final concentration of 5 units/ml at 4°C for 0.5 h and the released precursor PA was then used for *in vitro* processing.

Analysis of precursor PA processing in vitro. Routine incubations for monitoring *in vitro* processing were carried out at 23°C in 20 μl of 50 mM buffer at various pHs, containing 150 mM NaCl and purified precursor PA (20 μg/ml). The buffers used for this experiment were the following: pH 6.4, MES; pH 8.0, Tris; pH 9.8, 3-(cyclohexylamino) propanesulfonic acid (CAPS). After incubation, aliquots of the reaction mixture were mixed with an equal volume of sample buffer to stop the reaction, and analyzed by SDS–PAGE and immunoblotting.

Gel electrophoresis and Western blot analysis. SDS–PAGE was carried out by the method of Laemmli (16). Proteins were transferred from SDS–polyacrylamide gels to sheets of nitrocellulose (BA-S 85, 0.45 μm pore size, Schleicher and Schuell) by electroblotting. The nitrocellulose sheets were blocked with 5% skim milk in phosphate-buffered saline containing 0.1% Tween20 (PBST) for 1 h and then incubated with the polyclonal antibody directed against the large or the small subunit of penicillin acylase at room temperature. After washing three times with PBST, anti-rabbit IgG coupled to horseradish peroxidase was added for 1 h. After another three washes with PBST, the nitrocellulose sheet was developed with the ECL Western blotting analysis system (Amersham Pharmacia Biotech) to visualize the antigen antibody complexes.

Determination of penicillin acylase activity. The enzyme activity of PA was determined by the colorimetric assay described by Bomstein and Evans (17). The enzyme activity *in vivo* was calculated as A_{415} ($A_{600} \times$ reaction time (h)) $\times$ 100. The enzyme activities of wild-type and mutant PAs were the average of at least five independent assays.

RESULTS AND DISCUSSION

In Vitro Processing of Precursor PA

According to our earlier study, the deletion of signal peptide slows the processing of precursor PA (2). This result suggests that the precursor PA without signal peptide is a better substrate for monitoring *in vitro* processing. Therefore, a recombinant plasmid pGT-AATS, which encodes a GST-PA fusion protein, was constructed (Fig. 1A). Treatment of this fusion protein with thrombin could then release the precursor PA without signal peptide, which has three extra amino acids (Gly-Ser-Met) at its N-terminus. In the presence of IPTG, the fusion protein was induced in amounts of 5–10% of the total protein. However, most of the expressed fusion protein was insoluble. The fusion protein was further purified homogeneously from the soluble fraction by affinity chromatography as shown in Fig. 1B.

First, we tested whether the *in vitro* purified precursor shows the same processing phenotype as *in vivo*. As shown in Fig. 1C, the cleavage between the large subunit and the spacer peptide occurred first, and then the processing between the small subunit and the spacer peptide was followed by three stepwise cleavage reactions. These results demonstrated that the purified precursor PA is processed into active PA spontaneously the same pattern as *in vivo*, and also that the processing of the small subunit depends on the processing of the large subunit.

We could not exclude the possibility that the added thrombin affected the precursor PA processing *in vitro*. To examine this possibility, GST-PA fusion protein was

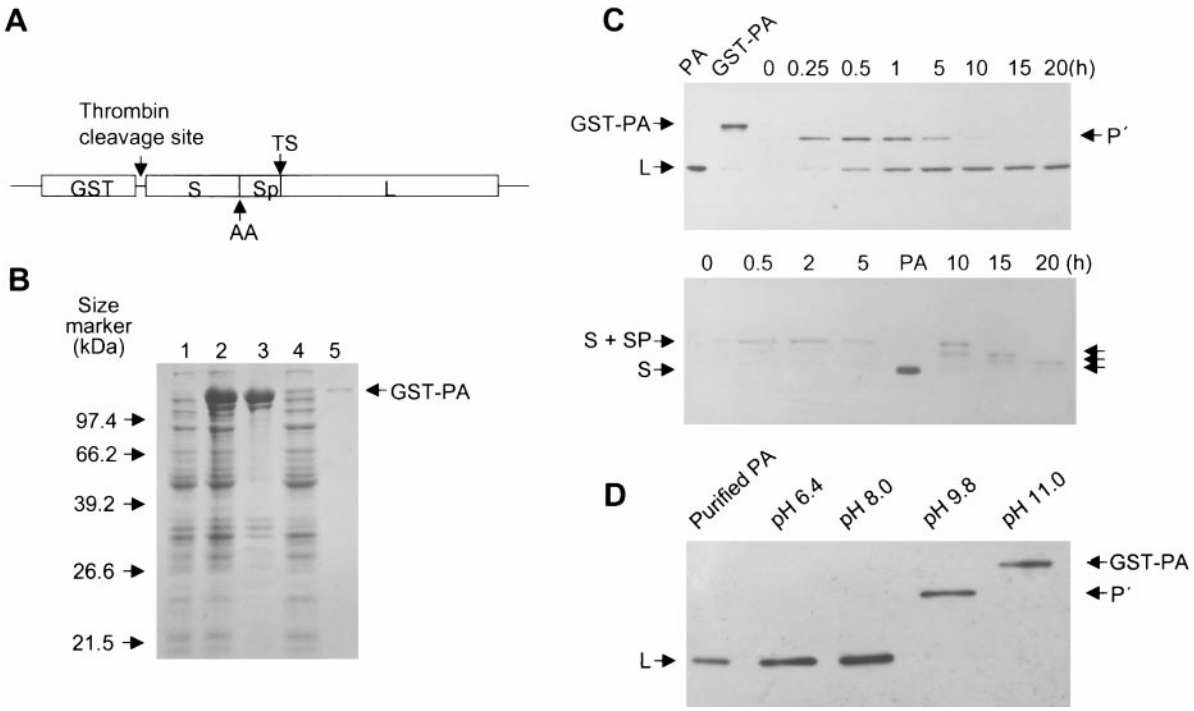

FIG. 1. Purification and *in vitro* processing of GST-PA fusion protein. (A) Schematic representation of translational protein fusion of PA without signal peptide to Glutathione-S-transferase (GST). S, small subunit; L, large subunit; Sp, Spacer peptide; AA, cleavage site between small subunit and spacer peptide (alanine–alanine); TS, cleavage site between spacer peptide and large subunit (threonine–serine). (B) SDS–polyacrylamide gel (10%) analysis of GST-PA precursor purification. Lanes: 1, 30 μg of total cell extract before induction; 2, 30 μg of total cell extract after induction with IPTG for 2 h; 3, 30 μg of insoluble proteins after induction; 4, 30 μg of soluble proteins after induction; 5, 0.5 μg of single-step purified GST-PA. (C) Time-dependent *in vitro* processing of precursor PA (P′). Upper panel: Processing of precursor PA (P′) into large subunit (L). Lower panel: Processing of small subunit-spacer peptide (S + SP) into small subunit (S). The arrows on the right indicate the processing intermediates. Large and small subunits were detected by polyclonal rabbit anti-L, anti-S antiserum/anti-rabbit-conjugated peroxidase. (D) pH dependent thrombin cleavage and precursor processing. Purified GST-PA was treated with thrombin protease of 5 unit/ml and incubated for 18 h at 23°C. The reactions were analyzed by Western blot.

incubated and processed with thrombin at various pH conditions. As shown in Fig. 1D, the efficiency of cleavage between the precursor PA and GST by thrombin was affected by pH. The thrombin was active at pH 9.8, but the precursor processing was blocked at pH 9.8. This result indicated that the *in vitro* processing of precursor PA was not affected by thrombin but by the pH of the reaction mixture.

Various reports suggest that intrinsic lysine or histidine residue is involved in the autocatalytic proteolysis of LexA and glycosylasparaginase (18, 19), and some serine protease have an active center in which both serine and lysine residues were involved (20, 21). These previous reports and pH dependency of precursor PA processing made us to try to identify another functional amino acid for the PA processing.

Identification of a Conserved Lysine Residue (K_{299}) in PA

The penicillin acylases from different species are similar to the glutaryl 7-aminocephalosporanic acid acylase (GL-7ACA) in their structure and enzymatical function and both PA from *E. coli* and GL-7ACA from *pseudomonas* species are matured by an autocatalytic processing pathway (12, 13). Therefore, we try to identify an active residue in the autocatalytic proteolysis by analysis of the primary amino acid sequence alignment with four PAs from gram negative bacteria, two PAs from gram positive bacteria, and two glutaryl 7-aminocephalosporanic acid acylases from *pseudomonas* species (4, 22–25). PA from *E. coli* shows about 60–85% amino acid sequence identity with gram negative bacteria, 28–39% identity with gram positive bacteria, and about 20% identity with GL 7-ACA from *pseudomonas.* This alignment revealed that there are two highly conserved clusters in PAs and GL 7-ACAs (Fig. 2). One of these regions is located at the N-terminal region of the small subunits, and the other at the N-terminal region of the large subunits. The N-terminal serine residue (S_{290}) that is essential for the enzyme catalysis and the precursor PA processing is absolutely conserved. It was also found that the lysine residue (K_{299}) at N-terminal region of the large subunit was highly conserved.

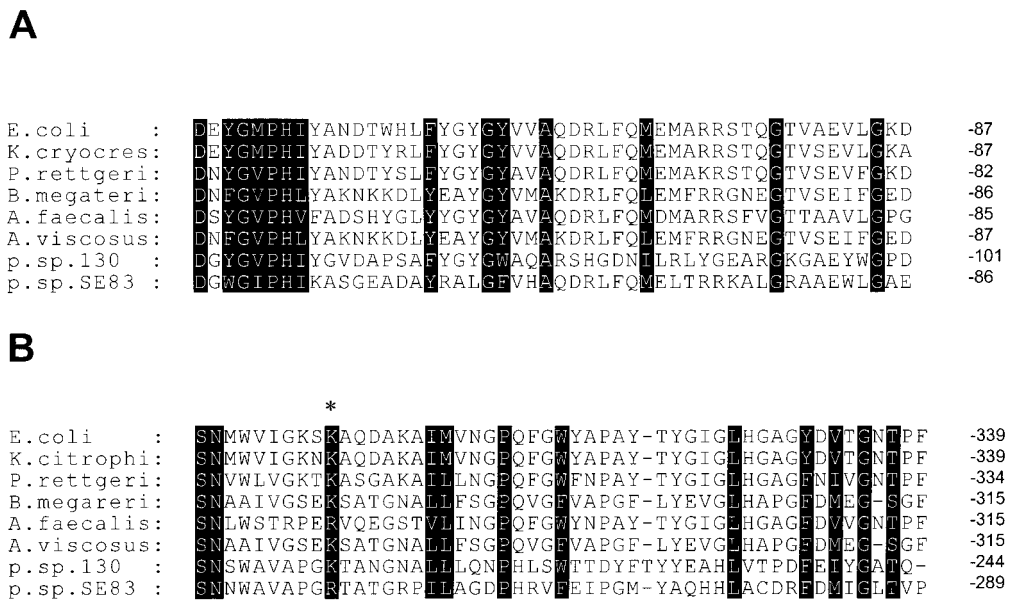

FIG. 2. Sequence alignment of six penicillin acylase and two GL 7-ACAs. Residues conserved among all proteins are shaded. (A) Conserved region in the small subunit. (B) Conserved region in the large subunit. Lysine denoted by asterisk (*) is a putative amino acid responsible for the pH dependent precursor PA processing. The Accession Numbers are: *Escherichia coli* (P06875); *Kluyvera crypcrescens* (P07941); *Providencia rettgeri* (1073664); *Bacillus megaterium* (1075790); *Arthrobactor viscosus* (P31956); *Alcaligenes faecalis* (U50186); *Pseudomonas* sp. 130 (AF085353); *Pseudomanas* sp. SE83 (P15558).

Considering the effect of the reaction pH on the precursor PA processing (Fig. 1D), lysine or arginine residue is the most probable amino acid, along with the N-terminal serine residue, responsible for the pH dependent processing. Therefore, this lysine residue was targeted for site-directed mutagenesis for its functional analysis of precursor PA processing.

The Effects of the Mutations at Lysine Residue (K_{299}) on the Precursor Processing and Enzyme Activity in Vivo

Site-directed mutagenesis based on the PCR was performed using the pPAKS63 vector containing *E. coli* penicillin acylase gene as a template. To test the possibility that this conserved lysine residue (K_{299}) is involved in the precursor PA processing, it was replaced by glutamine, serine, histidine, or arginine. As a result of PA enzyme assay and Western blot analysis (Fig. 3B), the K299H (the lysine replaced with histidine) mutant showed reduced PA activity, 10.2% of the wild-type, and slower processing rate than the wild-type precursor PA. But, two mutants, K299S (Lys to Ser) and K299Q (Lys to Gln), showed little enzyme activity, ranging from 0.2–0.4% of the wild-type enzyme activity, and almost none of the precursor was processed into the large subunit. In contrast, the enzyme activity of the K299R mutant (Lys to Arg) was the same as that of the wild type enzyme and most of the precursor is processed into functional mature PA. These results demonstrated that the processing efficiency of the

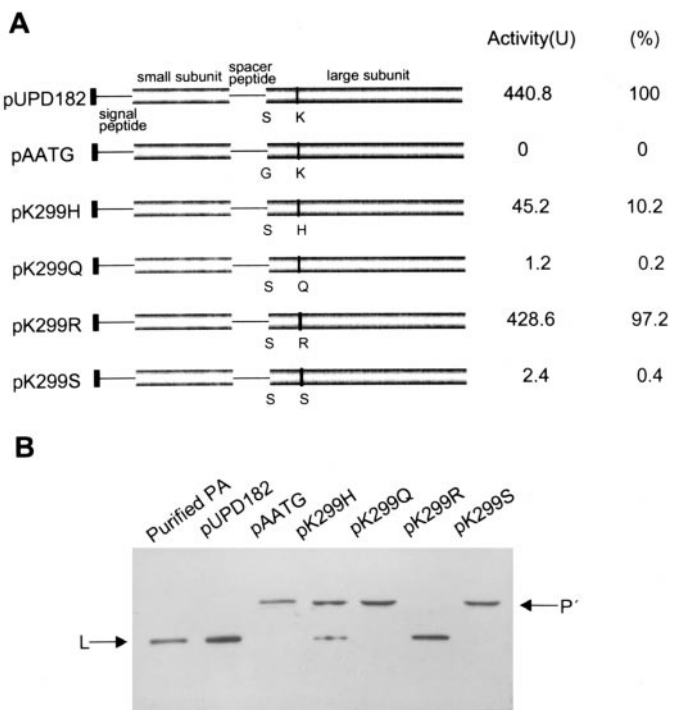

FIG. 3. Analysis of the site-directed mutants *in vivo.* (A) Schematic diagram and enzyme activity (U) of WT and mutant PA. Mutated amino acids are denoted as a single letter. (B) Western blot analysis of total cell lysates of *E. coli* $DH5_{\alpha}$ containing the WT and mutant plasmids. P′ and L denote the migration positions of precursor and large subunit, respectively.

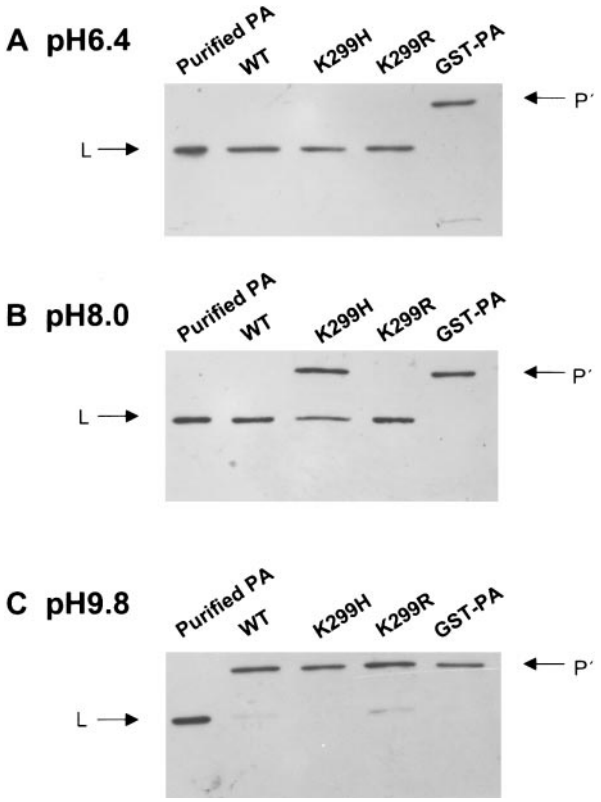

FIG. 4. pH-dependent *in vitro* processing of WT and mutant precursors. Purified precursors were incubated for 18 h at pH 6.4 (A), pH 8.0 (B), pH 9.8 (C). Samples were analyzed by SDS–PAGE (10%) and visualized by Western blot.

cleavage between the large subunit and the spacer peptide correlates with the enzyme activity. These *in vivo* data also support the idea that this conserved lysine residue (K_{299}) is important for the precursor processing.

Role of the Lysine Residue (K_{299}) in the Activation of Precursor PA Processing

It can be argued that the effect of site-directed mutagenesis on the processing of the precursor PA is not based on its chemical properties but on its conformational change. To clarify this possibility, the precursors PA of wild-type, His, or Lys subsituent (K299H, K299R) were processed *in vitro* for 18 h at various pH conditions (Fig. 4). At pH 6.4, wild-type and mutant precursors were perfectly processed into mature PA. At pH 8.0, most of the precursors of wild-type and K299R were processed into a large subunit. In contrast to the result at pH 6.4, the processing rate of the precursor of K299H was slower and a small amount of the processed form was detected as *in vivo.* This may reflect the fact that the physiological pH is close to pH 7.0. When the pH of the reaction mixture was increased to 9.8, the processing of all tested precursors was inhibited. The wild type and K299R showed much slower processing rates and only a small amount of precursors was processed. The K299H was perfectly blocked in precursor processing. The lysine, arginine, and histidine have a positively charged R group at neutral pH. Both lysine (pKa = 9.74) and arginine (pKa = 10.76) amino acids have pKa values over pH 9.5, but histidine (pKa = 7.59) is an amino acid having a side chain with a pKa near neutrality. Considering the chemical properties of the side chain and results from *in vitro* processing at various pH, the effect of mutations at lysine residue (K_{299}) on the precursor PA processing is not by structural change but through the chemical properties of its side chain R group. In a previous report by Sizmann *et al.* (5), it was shown that the deletion of C-terminal hexapeptide blocked the precursor PA processing. The authors suggested the possibility that C-terminal peptide, especially histidine residue, participated in the processing. But amino acid sequence analysis indicated that there was no meaningful homology among the acylases (Fig. 2). Furthermore, the replacement of C-terminal histidine residue (H_{864}) into proline could not inhibit the precursor processing (data not shown). Therefore, it was thought that the effect of C-terminal deletion on the processing resulted from a structural change.

In conclusion, we clearly demonstrated that S_{290} and K_{299} were critical amino acids for the precursor PA processing. These data strongly support the view that the maturation of precursor PA has occurred by a self-processing reaction via an active center consisting of S_{290} and K_{299}.

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