Expression and Characterization of the Peptidase Domain of *Streptococcus pneumoniae* ComA, a Bifunctional ATP-binding Cassette Transporter Involved in Quorum Sensing Pathway*

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ComA, a member of the bacteriocin ATP-binding cassette transporters, is postulated to be responsible for both the processing of the propeptide ComC and secretion of the mature competence-stimulating peptide, which regulates the competence and subsequent genetic transformation in *Streptococcus pneumoniae*. A recombinant N-terminal peptidase domain of ComA, designated PEP, was expressed as a soluble protein in *Escherichia coli*, purified to homogeneity, and characterized. Gel-filtration analysis revealed that PEP functions as a monomer. The purified PEP exhibited an efficient proteolytic activity for the substrate ComC, which was cleaved after the double glycine site. The stability of PEP was examined by circular dichroism analyses. A convenient method for analyzing the proteolytic activity of PEP was developed, and the kinetic parameters for ComC were determined ($k_{\text{cat}} = 1.5 \pm 0.083 \text{ min}^{-1}$ and $K_m = 62 \pm 9.0 \mu M$). Replacements of Cys17 of PEP with Ser or Ala and His96 with Ala resulted in complete loss of activity, indicating that both Cys17 and His96 are essential for the catalysis. Together with information from a protease data base, the N-terminal domain of ComA was concluded to belong to the same clan as the papain-like cysteine proteases. Mutant substrates, in which each of the double glycines was replaced with Ala, were cleaved very poorly by PEP. The mechanism of this strict substrate specificity is discussed on the basis of the sequence alignment with other cysteine proteases.

Quorum sensing (1) is a way that bacteria communicate with each other to respond properly to growth conditions and successfully survive as a "community." Bacterial cells are continuously releasing various chemical substances called autoinducers into their surrounding environment. As the population density of a bacterial species increases, so does the cognate autoinducer concentration, which subsequently reaches a threshold to bind to either the cell surface or intracellular receptors (2, 3). The signal pathway then becomes activated, which leads to a cascade of intracellular biochemical signals or altered gene expressions in the target bacteria. Many bacteria are known to regulate diverse physiological processes through this system, such as biofilm formation (4), regulation of sporulation (5), virulence factor expression (6), and activation of biofilm formation (7).

The competence-stimulating peptide (CSP) of *Streptococcus pneumoniae* is one of the well studied examples of autoinducers of Gram-positive bacteria. The 17-amino acid CSP is postulated to be cleaved from the 41-amino acid propeptide ComC and concomitantly exported by ComA with the help of an accessory protein, ComB (8). The accumulated CSP binds to the cell surface receptor ComD, which subsequently phosphorylates ComE by its histidine kinase activity and then induces the transcription of genes, such as comX and comW, associated with the DNA uptake (competence) and recombination (7, 9). Thus, ComA is a key molecule essential for the first step of the quorum-sensing system of *S. pneumoniae*.

ComA is a member of a family of bacteriocin-associated ATP-binding cassette transporters, which are composed of three domains (10): an N-terminal domain that has been proposed to possess the peptidase activity, a transmembrane domain consisting of six membrane-spanning segments, and a C-terminal ATP-binding domain located on the cytoplasmic face of the membrane. The peptidase domains of this family are thought to cleave their cognate propeptides after the consensus Gly-Gly motif. Recently, the peptidase domains of the family members LagD, a transporter of lactococcin G in *Lactococcus lactis*, and CvaB, a transporter of colicin V in *Escherichia coli*, were confirmed to have proteolytic activity, for which a cysteine residue is critical. However, both peptidase domains were expressed mainly as inclusion bodies in *E. coli*, and their biochemical characterizations remain limited (10, 11).

We now describe the high yield expression and purification of the N-terminal peptidase domain (PEP) of ComA, which enabled us to do detailed enzymological and protein-chemical characterizations of PEP. To the best of our knowledge, this is the first report describing the biochemical characterization of the signal-producing peptidase in the quorum-sensing pathway of Gram-positive bacteria.

**EXPERIMENTAL PROCEDURES**

**Cloning and Plasmid Constructions—** *S. pneumoniae* (ATCC 49619) was cultured in heart infusion broth (BD Biosciences) overnight at 37 °C, and the genomic DNA was isolated using a Genomic DNA kit (Qiagen, Hilden, Germany). The *comA* gene was amplified from the genomic DNA by PCR using the oligonucleotide primers 5′-GACTG-TACGCATATGAAATTTGGGAAACGTCACTATCGTCCGAAGCTGCA-CATC-3′ and 5′-ACTGTACGTCGACCTAGCTATGTGAACCAATTGGCGTTAAGGCTTCCCTCCT-3′. The underlined sequences represent the Ndel and SalI restriction sites. The PCR product was digested with Ndel and SalI and ligated into pPET-21b. Four independent clones were sequenced, and two silent mutations were found in all of the clones at Val52 (GTT to GTC) and Ala70 (GGG to GCA) in the peptidase domain of *comA*, compared with the sequence in the GenBankTM (M36180). The resultant plasmid was used as a template to amplify the CHispep3 fragment by PCR using the primers 5′-GACTGTCGACCATATGAA-lactopyranoside; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PEP, peptidase domain of *S. pneumoniae* ComA.
TTTGGGAAAACGTCACTATCGTCGCAAGT-3’ and 5’-GACTG-TACGGTGCCCATAGTGGGTTGTTGTTGCGCTTTGACATGGGCTTATAGTCT-3’. The underlined sequences represent the Ndel and Sall sites, and the italicized sequence encodes a His6 tag. After the amplification, the PCR product was digested with Ndel and Sall and ligated into pET-21b to generate the pSPP1.

The comC gene was also amplified from the *S. pneumoniae* genomic DNA by PCR using the primers 5’-GACTTGACCCATATGACCA-CCACACCAACAAAACAGTTAATGAGGACATG-TAGCTTTTGA and 5’-ACTGTCAGTGACCTATCTTTTTTCTTTGTTAAAATACAGCGGA-3’. The underlined sequences represent the Ndel and Sall sites, and the italicized sequence encodes a His6 tag. After the amplification, the PCR product was digested with Ndel and Sall and ligated into pET-21b to generate pSPP1. ComC* indicates ComC with a His tag on the N terminus.

The sequences of the coding regions of the expression plasmids were verified using an Applied Biosystems DNA sequencer, model 377.

**Site-directed Mutagenesis**—Site-directed mutagenesis was done using a QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions with pSPP1 as the template. The following primer pairs were used: 5’-GTTGAATCAGAGTGACCGTGTATGCTATTATCAAAGGCA-3’ (astersisks indicate mismatches) and 5’-TAATGAGCTACCGGCGCAGTATGACACAGCCACGAGCCAGGCA-3’. The complementary strand is the same as above except for the underlined sequences.

**Expression and Purification of ComCs**—For expression of the wild-type and mutant ComCs, an *E. coli* strain, JM109 (DE3) pLysS, was used. The *E. coli* cells carrying each expression plasmid were grown overnight at 37 °C in LB medium containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol. The overnight culture, 1.5 ml, was used to inoculate 300 ml of LB medium containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol, and the cells were grown with shaking at 37 °C for 3.5 h. For induction, IPTG was added to the culture to a final concentration of 0.2 mM, and the culture was continued at 37 °C for an additional 1 h.

After the expression, the cultured medium was immediately chilled in a ice water bath, and the cells were harvested by centrifugation and resuspended in 12 ml of a buffer (20 mM Tris-HCl, 500 mM NaCl, and 5 mM imidazole, pH 7.9) containing one tablet of the Complete mini protease inhibitor mixture (Roche Applied Science). The cell suspension was stored at −80 °C.

For purification, 12 ml of the cell suspension was thawed in a water bath, and any insoluble material was removed by centrifuging at 15,000 × g for 20 min at 4 °C. The supernatant was applied on a column packed with 1 ml of His-Bind column (Novagen, Madison, WI). After the column was changed using a PD-10 column (GE Healthcare) containing ribonuclease A (13.7 kDa), chymotrypsinogen A (25.0 kDa), and catalase (232 kDa). The elution of protein was equilibrated with 20 mM Tris-HCl, pH 7.0, and then passed through a Mono Q HR 5/5 column (GE Healthcare) connected to an ÄKTA FPLC system at a flow rate of 1.0 ml/min. The Mono Q column was pre-equilibrated and run with 20 mM Tris-HCl, pH 7.0. ComC* was recovered in the flow-through fraction. All chromatography procedures were done at ambient temperature. Routinely, ~1.5 mg of purified protein was obtained from 300 ml of bacterial culture. The purified ComC* were preserved at 4 °C for characterization.

**Expression and Purification of PEPs**—For expression of the wild-type and mutant PEPs, an *E. coli* strain, BL21 (DE3) pLysS, was used as the host. The *E. coli* cells carrying each plasmid were grown overnight at 37 °C in LB medium containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol. 5 μl of the overnight culture was used to inoculate 1 liter of LB medium containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol. The 5 μl of the overnight culture was used to inoculate 1 liter of LB medium containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol, and the cells were grown with shaking at 37 °C for 1.5 h. For induction, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.2 mM, and the culture was then allowed to grow at 30 °C for an additional 5 h.

After the 5-h induction, the cultured medium was chilled in an ice-water bath, and the cells were harvested and resuspended in 40 ml of a buffer containing 20 mM Tris-HCl, 500 mM NaCl, and 5 mM imidazole, pH 7.9. The cell suspension was divided into two and stored at −80 °C. For protein purification, 20 ml of the cell suspension was thawed in a water bath and then briefly sonicated, and any insoluble material was removed by centrifuging twice at 12,000 × g for 20 min at 4 °C. The supernatant was loaded on a column packed with 3 ml of His-Bind Resin (Novagen, Madison, WI). After the column was dialyzed against 20 mM Tris-HCl, 200 mM ammonium sulfate, and 2 mM dithiothreitol (DTT) at 4 °C and purified further using a Superose 12 10/300 GL column (GE Healthcare) connected to an ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare) at the flow rate of 0.5 ml/min. The column was pre-equilibrated with a running buffer, 20 mM Tris–HCl and 200 mM ammonium sulfate, pH 7.0. All chromatography procedures were done at ambient temperature. Routinely, ~10 mg of the purified protein was obtained from 500 ml of the bacterial culture for each of the wild-type and mutant PEPs. After DTT was added to a final concentration of 2 mM, the purified PEPs were preserved at 4 °C for characterization.

**Streptococcus pneumoniae ComA Peptidase Domain**

The sequences of the coding regions of the expression plasmids were verified using an Applied Biosystems DNA sequencer, model 377. The sequences of the coding regions of the expression plasmids were verified using an Applied Biosystems DNA sequencer, model 377. The sequences of the coding regions of the expression plasmids were verified using an Applied Biosystems DNA sequencer, model 377.
Circular Dichroism (CD) Measurements—The CD spectra of PEP were recorded using a Jasco spectropolarimeter, model J-720W1 (Jasco, Tokyo, Japan) equipped with a thermostat using a 0.1-cm light path sample cell. The thermal denaturation profiles of PEP were measured by monitoring the CD at 222 nm in the temperature range from 15 to 75 °C with the rate of temperature change 1 °C/min. The buffer was 30 mM sodium phosphate, 20 or 150 mM ammonium sulfate, and 0.2 mM DTT, pH 7.0.

For the pH stability experiments, PEP in 10 mM sodium phosphate, 200 mM ammonium sulfate, and 2 mM DTT, pH 7.0, was diluted with 9 volumes of the following buffer solutions: 30 mM sodium acetate (for pH 4.0 and 5.0), 30 mM sodium phosphate (for pH 5.8, 6.8, and 7.5), or 30 mM sodium borate (for pH 8.5, 9.0, and 9.2) containing 144 mM ammonium sulfate. The final pH values were determined after CD measurements using a Horiba pH meter, model F-8 (Horiba, Kyoto, Japan). The CD spectra were measured at 25 °C.

Enzyme Assay—The PEP activity was assayed in a 100-μl reaction mixture containing 50 mM potassium phosphate, pH 7.0, 150 mM ammonium sulfate, and various concentrations of the substrate. The reaction was started by adding the PEP solution to a final concentration of 0.25 μM. The reaction was carried out at 25 °C and quenched by freezing the mixture in liquid nitrogen. The reaction mixtures were thawed and loaded immediately onto a Waters μ Bondaspher C₈ reversed-phase column (3.9 × 150 mm, 100 Å pore size, 5-μm particle diameter) (Waters, Milford, MA) connected to a Beckman System Gold high performance liquid chromatography (HPLC) system (Beckman, Fullerton, CA), and the peptides were separated on a linear gradient from 10 to 55% (v/v) of acetonitrile containing 0.1% trifluoroacetic acid over 10 min at a flow rate of 1 ml/min at ambient temperature. The peptides were detected by monitoring the absorbance at 210 nm.

For the semiquantitative analysis to study the effects of the salts, pH values, or inhibitors on the PEP activity, the reaction mixtures were loaded on 16% SDS-polyacrylamide gel slabs. After the gels were stained with Coomassie Brilliant Blue R-250, the amounts of the products were compared visually among the lanes.

RESULTS

Overexpression and Purification—The N-terminal 150-amino acid domain of ComA, designated PEP (Fig. 1A), was expressed in E. coli, and the soluble fraction of the lyase was checked by SDS-PAGE. The amount of soluble PEP was increased significantly when expressed at 30 °C, compared with that when expressed at 37 °C (Fig. 1B). The soluble PEP significantly decreased at 25 °C (not shown), and thus the expression for the PEP purification was done at 30 °C. PEP was purified by a simple two-step procedure to apparent homogeneity as judged on a SDS-polyacrylamide gel. The N-terminal sequence of the purified PEP was determined as Met-Lys-Phe-Gly-Lys (performed by Nippi (Tokyo, Japan)), which corresponded to the predicted N-terminal sequence of PEP.

Subunit Structure—The purified PEP was eluted by gel filtration as a single, symmetrical peak. The Kᵥₑᵥₑ value approximately corresponds to 23.8 kDa based on the standard curve with known proteins (Fig. 1C). Because the theoretical molecular mass of PEP is 17.9 kDa, this result suggests that PEP exists as a monomer.

Structural Stability—Fig. 2A shows the CD spectra of PEP in the far-UV region from 190 to 300 nm measured at 15 °C and at 75 °C. The spectrum at 15 °C is typical of the unfolded, native state of a protein. At 75 °C, the spectrum showed drastic changes, and the solution became turbid, indicating that PEP was denatured. The denaturation was irreversible, and therefore, the following thermal-denaturation profiles are only semiquantitative and do not provide exact thermodynamic parameters. The CD value at 222 nm was monitored while the PEP solution was heated from 15 to 75 °C (Fig. 2B). The temperature scan in the presence of 20 mM ammonium sulfate showed a denaturation curve with an apparent Tᵥₑᵥₑ value of 42.5 °C. The Tᵥₑᵥₑ value increased to 47.9 °C in 150 mM ammonium sulfate. PEP is thought to be stable from 15 to 30 °C under both conditions.

Fig. 2C shows the CD values at 222 nm for different pH values measured immediately and at 24 h (kept at 25 °C) after the pH changes. The zero time data show that PEP is stable in the pH region of 6.8–9.0. After 24 h, small differences in the CD values at 222 nm were observed at pH values from 6.8 to 9.0. This might be explained by a simple dilution effect.
FIGURE 2. CD measurements of PEP. The ordinates indicate the mean residue ellipticity. The CD measurements were done at a protein concentration of 17.7 μM (A and B) or 8.85 μM (C). A far-UV CD spectrum of PEP in the region from 190 to 300 nm of PEP in the presence of 20 mM ammonium sulfate at 15 °C (1) or 75 °C (2). B, thermostability of PEP. Thermal denaturation of PEP was done in 30 mM sodium phosphate, pH 7.0, 0.2 mM DTT, and 20 mM (1) or 150 mM (2) ammonium sulfate. Thermal denaturation was monitored by measuring the CD changes at 222 nm while heating the sample at the rate of 1 °C/min. C, pH stability of PEP. The CD values at 222 nm and 25 °C immediately (○) and at 24 h (△) after the pH shifts to 4.0, 5.0, 5.8, 6.8, 7.5, 8.5, 9.0, and 9.2. The PEP stock solution in 10 mM sodium phosphate, 200 mM ammonium sulfate, and 2 mM DTT, pH 7.0, was diluted with 9 volumes of the buffer solutions described under “Experimental Procedures.”

FIGURE 3. Separation and detection of ComC* and its proteolytic products. A, amino acid sequence of ComC*. A His6 tag is attached to the N terminus of ComC. Numbers represent the amino acid residue numbers. The arrowhead indicates the processing site of ComC* by PEP. B and C, HPLC analysis of ComC* with or without PEP treatment, respectively. 100 μM ComC* was incubated with 1 μM PEP in 100 μM of 50 mM potassium phosphate, 150 mM ammonium sulfate, 0.2 mM DTT, and pH 7.0, at 25 °C. The reaction mixtures were loaded onto a Waters µBondosphere C8 reversed-phase column connected to a Beckman System Gold HPLC system, and the peptides were separated on a linear gradient from 10 to 55% (v/v) acetonitrile containing 0.1% trifluoroacetic acid over 10 min at a flow rate of 1 ml/min at ambient temperature. The retention time of ComC* (peak 1 in B and C) is 9.4 min, and those of the proteolyzed products (peaks 2 and 3 in C) are 7.7 and 9.0 min, respectively.

FIGURE 4. v/[E] versus substrate concentrations plot of PEP peptidase activity for ComC*. Different concentrations of ComC* were digested with 0.25 μM PEP in 50 mM potassium phosphate, 150 mM ammonium sulfate, and 0.2 mM DTT, pH 7.0, at 25 °C. Time courses of the increase in the product (peak 2) at concentrations of ComC* are shown in the inset (○, 12.5 μM; □, 25.0 μM; ●, 50.0 μM; A, 75 μM; ◆, 100 μM; □, 150 μM; and ○, 200 μM ComC*). The reaction rate (v) was divided by the enzyme concentration and plotted versus the ComC* concentration. The data were fitted with a saturation curve with the parameters of Kcat = 1.5 min⁻¹ and Km = 62 μM.

To quantify the reaction products, the HPLC method described under “Experimental Procedures” was employed. Fig. 3B shows the elution pattern of ComC*. Minor peaks are contaminants of the purified ComC* and did not affect the following analysis. After a 2-h PEP reaction, ComC* was converted into two products (Fig. 3C). The molecular weight of peak 2 was 3,608, and that of peak 3 was 2,244, indicating that they are proteolyzed products derived from ComC* whose molecular weight was determined as 5,793 by MALDI-TOF mass spectrometry analysis. The N-terminal sequence of peak 3 was determined as Glu-Met-Arg-Leu-Ser, which corresponds to the reported sequence of S. pneumoniae CSP (8). Thus, the cleavage site was identi-
fied as shown in Fig. 3A. The theoretical molecular weights of the two products are 3,567 and 2,244, which are close to the measured values for peaks 2 and 3, respectively. The molecular mass of peak 2 was 41 Da greater than the theoretical value. This might be because a small molecule such as a salt ion is attached.

Kinetic Parameters for ComC*- The enzymatic activity was evaluated by the rate of formation of the products. Because the decrease in the area of the substrate (peak 1) was always equal to the sum of the increases in the area of the products (peaks 2 and 3), the amounts of the products were determined from a standard curve of peak 1. The molecular mass of peak 2 was 41 Da greater than the theoretical value. This might be because a small molecule such as a salt ion is attached.

To confirm the protease type of PEP, Cys17 was replaced with Ser or Ala, and His96 was replaced with Ala. Cys17 is the only cysteine residue of PEP, and His96 is proposed to make a hydrogen bond with Cys17 (discussed below). None of these mutants showed proteolytic activity (<0.1% of the wild-type enzyme), indicating that Cys17 and His96 are essential residues for the catalysis, and PEP was confirmed as a cysteine protease.

Effects of Protease Inhibitors—The PEP peptidase activity was not sensitive to known protease inhibitors not only for the aspartate, metallo, or amino peptidases (pepsstatin at 150 μM, Pefabloc SC at 4 mM, chymostatin at 3.3 mM, aprotinin at 15 μM, EDTA at 5 mM, phosphor- amidon at 3 mM, and bestatin at 1.3 mM), but also those for the serine/cysteine proteases, (antipain at 1 mM and leupeptin at 1 mM) and cysteine proteases, (E-64 at 5 mM).

Activity for Mutant ComC*- The double glycin sequence was mutated to investigate the substrate recognition of PEP. Each of the two glycines was replaced with alanine, and the activities of PEP for them were compared with that for the wild-type ComC*. The kcat/Km values for Gly1Ala (Gly-Ala-) and Gly2Ala (Ala-Gly-) decreased to 0.12% and 0.22% of that for ComC* (Gly-Gly-), respectively (Table 1).

DISCUSSION
ComA is a membrane protein, and as expected, we could not express the full-length protein in E. coli (not shown). The N-terminal putative peptidase domain of ComA (PEP) could, however, be expressed as a soluble protein. Early in the course of the purification, PEP was found to be precipitated during dialysis against buffers with low salt concentrations. Also, the activity could not be detected at 37 °C under low salt conditions. Among the various tested salts, ammonium sulfate showed a significant positive influence on the PEP activity. Therefore, ammonium sulfate was added to the buffers throughout the dialysis and gel filtration steps and for the PEP assay. The expression level of PEP was improved significantly at 30 °C. All of these findings can be explained by the CD measurement results: PEP is quickly denatured at 37 °C under low salt conditions, and ammonium sulfate stabilizes PEP. The activity of the peptidase domain of LagD was increased by sodium sulfate (10), and it also improved the PEP activity detected by the semiquantitative SDS-PAGE assay. Because sodium sulfate was found to interfere with the HPLC assay, ammonium sulfate was employed in the present study.

Although the activity of the peptidase domain of CvaB was enhanced by Ca2+ (11), the PEP activity was not influenced by Ca2+. The PEP activity was not affected either by NaCl, KCl, MgCl2, or Mg2+-ATP. The PEP activity was also examined in Tris-HCl or potassium phosphate buffers in the pH region 6.0–8.8, and the highest activity was observed in 50 mM potassium phosphate, pH 7.0.

Peptidases are usually assayed using amino acids or peptides conjugated with chromophores such as p-nitroaniline, β-naphthylamide, and 4-methylcoumaryl-7-amide (13, 14). To establish an experimental system to elucidate the recognition mechanism of PEP for natural substrates, His-tagged ComC* was prepared, and the HPLC assay system was developed to detect and quantify ComC* and the proteolyzed products. The present results clearly show that ComA indeed possesses an efficient peptidase activity in its N-terminal domain, cleaving ComC at the double glycin site to generate CSP. PEP exhibited kcat and Km values of 1.5 min⁻¹ and 62 μM for ComC*, respectively. Although these parameters are probably influenced by other parts of the ComA protein including the ATP-binding domain, they would be in a reasonable range to produce a quorum-sensing signal, which is effective in nanomolar concentrations (8).

In this study, PEP has been experimentally demonstrated to be a cysteine protease. In the MEROPS database (merops.sanger.ac.uk/), ComA of S. pneumoniae has been classified in the same clan as the papain-like cysteine proteases (15). The active site catalytic triad Cys17, His96, and Asp112 and the oxyanion hole Gln11 of PEP are completely

**TABLE 1**

Activities of PEP for wild-type and mutant ComC*

| ComC*             | kcat  | Km   | kcat/Km |
|-------------------|-------|------|---------|
|                   | min⁻¹ | μM   | μM⁻¹    |
| ComC*             | 1.5 ± 0.083 | 62 ± 9.0 | 400     |
| Gly1Ala           | b     | b    | 0.48 ± 0.053 |
| Gly2Ala           | b     | b    | 0.89 ± 0.090 |

* Gly1Ala and Gly2Ala are mutant ComC*, where the glycine residue at the P1 or P2 site was replaced with alanine, respectively.

* Reactions did not show saturation kinetics at the examined substrate concentrations.

**FIGURE 5.** Sequence alignment of PEP of ComA with papain-like proteases. PEP of ComA was aligned with the peptidase domains of LagD and CvaB, ubiquitin C-terminal hydrodolase (YUH1), glycylic endopeptidase, staphopain A, cathespin B, and papain. The first and last residue numbers are amino acid positions. The numbers of excluded residues are in parentheses. Hyphens are gaps in the alignment. The conserved active site catalytic triad, Cys-His-Asp/Asn, and the oxyanion hole glutamate residue are marked with asterisks. Residues corresponding to the S1 and S2 subsites are indicated by 1 and 2, respectively.
conserved in a sequence alignment with other papain-like cysteine proteases (Fig. 5). In the cysteine proteases, the Cys and His residues are thought to be the catalytic residues that act as a proton donor and its acceptor, respectively. Besides the Cys and His residues, two residues are also important for catalysis in the papain-like proteases. These are a Gln residue that helps in the formation of the oxyanion hole, an electrostatic center that stabilizes the tetrahedral intermediate, and an Asp or Asn residue that is thought to orient the imidazole ring of the catalytic His, creating the catalytic triad (16). The Cys17 and His96 mutants of PEP showed a complete loss of activity, which supports the idea that Cys17 and His96 are the essential amino acid residues for the proteolytic activity.

The substrate specificity of PEP was found to be very strict because even a minimum change (Gly to Ala) at the P1 or P2 subsite of ComC* was not tolerated by PEP. Unlike protein-degrading proteases, the strict substrate specificity of PEP would be a prerequisite for the signal-producing activity of ComA. Consistent with this, the PEP activity was not sensitive to typical peptide-mimetic serine/cysteine or cysteine protease inhibitors, such as antipain, leupeptin, and E-64, which inhibit papain or cathepsin B (17).

There are several papain-like proteases that specifically recognize a glycine residue in their substrates. To date, some of their three-dimensional structures have been elucidated: for example, ubiquitin C-terminal hydrolase, which digests substrate proteins after Gly. In these proteases, residues with bulky side chains occupy the corresponding subsite(s) and make a tight space to fit glycine(s) of the substrate (Fig. 5) (18, 19). On the contrary, the S1 or S2 subsite of papain or other family members is a wide, unrestricted pocket that accommodates a relatively wide variety of substrates (20). From the sequence comparison, rather bulky side chains of Met15 and Tyr99 in PEP are predicted to construct the S1 and S2 subsites, respectively, where Gly and Ala residues are present in papain, staphopain A, and cathepsin B. This might confer PEP with its strict requirement of glycines at both the P1 and P2 sites of the substrate, ComC. In CvaB, which also recognizes the double glycine site, Ala is predicted to occupy the S1 subsite from the sequence alignment. This might explain why CvaB showed proteolytic activity for L-arginine-p-nitroanilide and partial susceptibility to antipain (11). In the future, the crystallographic analysis of PEP will provide a clear answer regarding its unique substrate specificity.

The present results would not only open new possibilities to study the quorum-sensing system of S. pneumoniae, but also provide an ideal model to study the peptidase domains of bacteriocin-associated ATP-binding cassette transporters.

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