Characterization of a Unique Glycosylated Anchor Endopeptidase That Cleaves the LPXTG Sequence Motif of Cell Surface Proteins of Gram-positive Bacteria*

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The precursors of most surface proteins on Gram-positive bacteria have a C-terminal hydrophobic domain and charged tail, preceded by a conserved LPXTG motif that signals the anchoring process. This motif is the substrate for an enzyme, termed sortase, which has transpeptidation activity resulting in the cleavage of the LPXTG sequence and ultimate attachment of the protein to the peptidoglycan. While screening a group A streptococcal membrane extract for cleavage activity of the LPXTG motif, we identified an enzyme (which we term “LPXTGase”) that differs significantly from sortase but also cleaves this motif. The enzyme is heavily glycosylated, which is required for its activity. Amino acid composition and sequence analysis revealed that LPXTGase differs from other enzymes, in that the molecule, which is about 14 kDa in size, has no aromatic amino acids, is rich in alanine, and is 30% composed of uncommon amino acids, suggesting a nonribosomal construction. A similar enzyme found in the membrane extract of Staphylococcus aureus indicates that this unusual molecule may be common among Gram-positive bacteria. Whereas peptide antibiotics have been reported from bacillus species that also contain unusual amino acids and are synthesized non-ribosomally on amino acid-acetylating polynucleotide templates, this would be the first reported enzyme that may be similarly synthesized.

A large group of cell surface proteins of Gram-positive bacteria are covalently anchored through their C termini to the cell wall peptidoglycan. Most of these proteins are essential for pathogenic bacteria to establish successful infection of host tissues, and hence they are considered virulence factors. Functionally, these surface proteins may be divided into three major groups: viz. 1) those with adhesin or invasion function (1–27); 2) those with antiphagocytic activities (28–40); and 3) those that are enzymes that degrade surface components of host cells, thereby facilitating spread, and enzymes that hydrolyze large molecules in the surroundings into utilisable nutrients (29, 41–48).

A striking feature of all of these functionally and structurally diverse surface proteins is that they all possess a carboxyl-terminal LPXTG sequence (49), which is cleaved during surface translocation at the septum (50), resulting in a covalent linkage to cell wall peptidoglycan. In all cases, the genes for these proteins contain additional nucleotide sequences following that which encodes the LPXTG. These additional sequences encode a stretch of hydrophobic amino acids and positively charged C-terminal amino acids. Pancholi and Fischetti (51) observed that the hydrophobic and positively charged amino acid sequences are missing in the cell wall-linked M protein, indicating that the precursor of M protein was cleaved at a site within or immediately C-terminal to the LPXTG sequence. These findings strongly indicated that surface proteins become anchored to the cell wall by a common mechanism (49). Subsequently, it was shown that deletion of either the LPXTG or hydrophobic amino acid sequence or charged terminal amino acid from the precursor of protein A of S. aureus results in failure of protein A anchoring to the cell wall (52), indicating that these sequences were essential for the cell wall-anchoring process of these proteins. Collectively, these sequences are considered to be a cell wall sorting signal, which has now been shown to be present in over 100 surface proteins of Gram-positive bacteria (53, 54).

Through a series of elegant experiments, Schneewind et al. (55) have shown that the peptide bond between threonine and glycine of the LPXTG sequence of protein A becomes cleaved by an enzyme termed sortase, after which the carboxyl-terminal terminus of threonine becomes covalently attached to the amino group of one of the glycines of the pentaglycine cross-bridge of the S. aureus cell wall. Recently, they have shown that S. aureus mutants defective in the anchoring of surface proteins to the cell wall carry a mutation in srt gene (56). Subsequently, they cloned the srtA gene in Escherichia coli and purified recombinant sortase. In vitro, the purified sortase cleaved the LPXTG sequence after threonine (56) and also covalently attached the surface protein with C-terminal LPXT to a triglycine substrate (57). These results indicate that sortase possesses two functions, a specific endopeptidase and a transpeptidase. In addition, they showed that S. aureus mutants lacking sortase are unable to display surface proteins and are defective in establishing infection (58). An analysis of the genome of several Gram-positive bacteria revealed that there is more than one sortase gene per bacterial genome (54).

In the present report, we have identified and purified an enzyme from Streptococcus pyogenes that actively cleaves the LPXTG anchor motif but is very different from the sortase of S. aureus in its glycosylation and presence of uncommon amino acids. We here describe the physical and biochemical properties of this enzyme, which we term LPXTGase.

**EXPERIMENTAL PROCEDURES**

Enzymes—N-Glycosidase F (catalog no. 1,365,193) and O-glycosidase (catalog no. 1,347,101) were purchased from Roche Molecular Biochemicals. β-N-Acetylhexosaminidase (A7708) and β-glucosidase (G6906)
were purchased from Sigma. Group C streptococcal C1 phage lysis was prepared by the method described by Nelson et al. (59).

**Other Materials**—Silica gel TLC plates (catalog no. M 5729-6), solvents, and other chemicals were purchased from Fisher. Carboxymethyl glass beads (G-3910) and 1-ethyl-3-(dimethylaminopropyl) carbodiimide (E-1769) and buffers were purchased from Sigma. 125I was purchased from PerkinElmer Life Sciences.

**Bacterial Strain and Culture**—S. pyogenes strain D471 was grown in Todd-Hewitt medium supplemented with 1% yeast extract in a fermenter. Cells were harvested when the OD at 650 nm reached 1.0. To harvest the cells, the culture was concentrated to about 2 liters in a fermenter. Cells were harvested when the OD at 650 nm reached 1.0. To harvest the cells, the culture was concentrated to about 2 liters

**Cell Lysis and Preparation of Crude Extract**—The washed cell pellets described above were suspended in 1.2 liters of 30 mM MES buffer, pH 6.2, and cell clumps were gently dispersed with a Dounce homogenizer.

**Preparation of LPXTG Peptide Substrate**—An LPXTG-containing peptide, KRQLPTSTGETANPFY from the streptococcal M6 protein, was synthesized, labeled, and purified in the same way.

**Enzyme Assay Method**—Aliquots (10 μl) of fractions were assayed for cleavage activity of bead-bound 125I-labeled LPXTG-containing peptide as described under “Experimental Procedures.” The solution eluting from the column in the fractions appeared clear and turbid.

**Optimization of Enzyme Activity**—To determine the pH optimum of enzyme activity, enzyme reactions were carried out under various pH conditions using the following buffers: MES-NaOH (pH 5, 6, 6.5, and 7), Tris-HCl (pH 7.5, 8.0, 8.5, and 9), NH4OH-HCl (pH 10), and triethylamine HCl (pH 11 and 12). To determine the effect of detergents on enzyme activity, enzyme reactions were carried out in the presence of 0.05–0.5% of Brij 35 or Triton X-100. To determine the optimal duration
of reaction time, the amount of radioactive peptide released from the beads was measured at 10-min intervals up to 2 h.

Identification of Enzyme Reaction Products—About 2 μg of bead-bound or free KRQLPSTGETANPFY, in which the terminal tyrosine was labeled with 125I, was incubated with purified LPXTGase in 50 μl of 30 mM Tris-HCl buffer, pH 7.6, containing 0.1% Brij in separate Microfuge tubes for a varying length of time at 37°C with vigorous shaking. For reactions with bead-bound peptide, the tubes were centrifuged at designated times in order to pellet unreacted bead-bound peptide, and 30-μl aliquots of the supernatant were spotted onto a silica gel TLC plate. For reaction with free peptide, 30-μl aliquots from each tube at designated times were directly spotted on a silica gel TLC plate. The plates were developed with a solvent mixture consisting of ethyl acetate/pyridine/acetic acid/water (60:30:9:24), and the reaction products were located by autoradiography. Reaction products were eluted from the plate, and amino acid sequences were determined with an Applied Biosystems AB1 Procise 494 instrument by the Rockefeller University protein chemistry laboratory.

Various concentrations of LPXTGase or trypsin (as control) was added to 50 μg of bovine serum albumin (U. S. Biochemical Corp.) under conditions described above and incubated for 1 h at 37°C. A sample of the reaction mixtures was analyzed by SDS-PAGE for degradation.

Enzyme Kinetics—Varying concentrations of 125I-labeled KRQLPSTGETANPFY peptide, ranging from 20 to 240 μM were incubated with 2.4 μM of the purified enzyme in 50 μl of 50 mM Tris-HCl buffer, pH 7.6, containing 0.1% Brij 35 at 37°C for 30 min. At the end of the reaction time, 30 μl of the reaction mixtures were spotted on silica gel TLC plates, and the plates were developed with ethyl acetate/pyridine/acetic acid/water (60:30:9:24). The plates were then autoradiographed, and substrate and reaction products were scraped off the plate and counted for radioactivity.

FIG. 2. Chromatography on Sephadex G50 of the clear fall-through material from the DEAE-cellulose column. The clear fall-through fractions eluting from the DEAE-cellulose column in Fig. 1 were pooled and concentrated by means of ultrafiltration. The concentrated enzyme solution was subjected to gel filtration on a Sephadex G50 column, and aliquots (10 μl) of fractions from the column were assayed for cleavage activity of bead-bound 125I-labeled LPXTG-containing peptide as described under “Experimental Procedures.”

FIG. 3. SDS-PAGE analysis of the 125I-labeled LPXTGase. The ethanol and ethyl acetate-precipitated enzyme that had been labeled with 125I-tyramine was solubilized in 0.2% SDS in 100 mM Tris-HCl buffer, pH 6.8, and after boiling, the enzyme was subjected to SDS-PAGE using 16% acrylamide gel, and the dried gel was autoradiographed.
Enzyme Concentration and Dry Weight Determination—The fractions with enzyme activity eluting from the Sephadex G50 column were combined, and the enzyme solution, amounting to about 150 ml, was concentrated to about 10 ml by YM3 ultrafiltration, after which an aliquot of 2–3 ml was lyophilized. The lyophilized enzyme was dissolved in 200 μl of distilled water, and the concentrated enzyme solution was divided into two preweighed Microfuge tubes. To each tube 300 μl each of ethanol and ethyl acetate was added. The tubes were kept at −20 °C, precipitating the enzyme, leaving most of the Brij 35 and buffer salts in the supernatant. The precipitated enzyme was pelleted by centrifugation, and the pellets were dissolved in a small volume of distilled water. This enzyme solution was used as the starting material for various chemical analyses. For dry weight determination, the precipitate was dissolved in 200 μl of distilled water and the ethanol/ethyl acetate

![Fig. 4. Cleavage of an LPXTG peptide by LPXTGase. A, bead-bound KRQLPSTGETANPFY, in which the C-terminal tyrosine was labeled with 125I, was incubated with purified LPXTGase, and the reaction product was examined on silica gel TLC as described under “Experimental Procedures.” B, free KRQLPSTGETANPFY, in which the C-terminal tyrosine was labeled with 125I, was incubated with purified LPXTGase, and the reaction products were examined on silica gel TLC as described under “Experimental Procedures.” Y, the location of free tyrosine. The locations of other peptide sequences were identified after sequencing the peptide within their respective spots.](image1)

**Fig. 5.** The kinetics of cleavage of LPXTG peptide by LPXTGase. 125I-Labeled KRQLPSTGETANPFY, ranging from 20 to 240 μM, was incubated for 30 min with 2.4 μM LPXTGase, and the kinetics of cleavage of the peptide was determined as described under “Experimental Procedures.”

**Fig. 6.** pH optimum of LPXTGase activity. The optimum pH for LPXTGase activity was determined, at 50 mM concentrations of various buffers. The buffers employed were as follows: MES-NaOH for pH 5, 5.5, 6, 6.5, and 7; Tris-HCl for pH 7.5, 8, 8.5, and 9; NH₄OH-HCl for pH 10; and triethylamine HCl for pH 11 and 12.
precipitation step was repeated two more times in order to remove residual detergent and salts, the final precipitate was dried in vacuo, and the tubes were weighed.

Amino Acid and Sugar Composition of LPXTGase—The amino acid and sugar composition of the LPXTGase were determined by the Rockefeller University Protein Chemistry Laboratory using a Waters 490 HPLC system. The concentrated enzyme in distilled water described above was used as starting material for these analyses.

**TABLE I**

| Salts                  | Concentration (mM) | Peptide cleaved (cpm) | Percentage of control (%) |
|------------------------|--------------------|-----------------------|---------------------------|
| Sodium chloride       | 100                | 6,720                 | 16                        |
| Potassium chloride    | 100                | 5,608                 | 20                        |
| Hydroxyamine HCl      | 100                | 11,200                | 27                        |
| Magnesium chloride    | 20                 | 5,615                 | 20                        |
| Calcium chloride      | 20                 | 9,706                 | 23                        |
| Putrescine chloride   | 20                 | 8,710                 | 21                        |
| Sodium phosphate      | 20                 | 20,646                | 49                        |
| Sodium sulfate        | 20                 | 23,454                | 57                        |
| EDTA                   | 20                 | 2,147                 | 5.2                       |
| Control (no salt)     |                    | 41,550                | 100                       |

**RESULTS**

Purification of the LPXTGase—Using the bead-bound [125I]labeled KRQLPSTGETANPFY peptide from *S. pyogenes*, which contains the LPXTG motif, we attempted to identify an endopeptidase in the streptococcal cell extract. In numerous initial trials, no LPXTG cleavage activity was detected in any crude fraction or in fractions from chromatography columns. Eventually, we discovered that ultrafilters of the crude extract using a YM3 filter removed a low molecular weight substance that inhibited the cleavage activity. After removing this inhibitor by ultrafiltration, active enzyme could be prepared from both cytosol and membrane extracts. The enzyme activity in the cytosol is entirely attributable to membrane vesicles released into the cytosol rather than a soluble cytosol fraction. For example, lysis treatment of *S. pyogenes* results in localized digestion of the cell wall, producing holes in the cell wall. Through these holes, segments of cell membrane exude externally, which become pinched off as vesicles (59). When the turbid cytosol was centrifuged for 1 h at 30,000 rpm, membrane vesicles along with all of the enzyme activity were pelleted. A large part of the cell membrane still remained associated with the cell ghosts, and more cleavage activity could be extracted from the ghost-associated membranes.

Preliminary experiments revealed that LPXTG cleavage activity does not bind to DEAE-cellulose, whereas most proteins did. Thus, the cytosol fraction containing membrane vesicles or the membrane extract of the cell ghost was directly applied to a DEAE-cellulose column as the first step of enzyme preparation. Free enzyme released from the membrane eluted first in the clear fall-through fraction, which was followed by the vesicle-containing turbid fraction. However, neither the clear nor turbid fall-through fractions showed an LPXTG cleavage activity initially (Fig. 1). Thus, when the volume of the clear fall-through fraction was reduced by ultrafiltration using a 3-kDa cut-off YM3 membrane, the retentate exhibited enzyme activity. This suggests that a low molecular weight inhibitor eluted from the DEAE column together with the enzyme in the fall-through fraction, and the inhibitor passed through the membrane during ultrafiltration. As the concentration of Brij 35 in the fall-through fraction increased during concentration, it formed micelles, which could not pass through YM3 membrane, and as a consequence the retentate became very viscous. Therefore, to remove Brij 35, a large volume of detergent-free Tris-HCl buffer was added to the retentate, and the ultrafiltration process was continued. During this procedure, more of the enzyme inhibitor passed through the YM3 membrane along with...
with monomeric Brij 35, increasing the activity of the enzyme in the retentate.

When the concentrated enzyme solution was subjected to gel filtration using Sephadex G50, an active enzyme peak eluted soon after the void volume (Fig. 2). The activity peak did not absorb UV at 280 nm, indicating that the enzyme does not contain aromatic amino acids. Comparison with the elution profiles of proteins of known molecular weights indicates that the apparent molecular weight of the cleavage enzyme is about 14,000.

SDS-PAGE analysis revealed no protein bands stained with either Coomassie Blue or silver, even when over 100 μg of the purified enzyme was applied to a 16% gel (not shown). However, the lack of protein bands verified the purity of the enzyme preparation. Thus, in order to detect the enzyme in polyacrylamide gels, 125I-labeled tyramine was linked to the carboxyl groups of the enzyme by means of carbodiimide catalysis, and the radioactive enzyme was subjected to SDS-PAGE. Autoradiography of dried gels showed that most of the labeled enzyme was retained in the stacking gel, and only a small amount entered the running gel (Fig. 3). When the enzyme was labeled with fluorescein isothiocyanate and subjected to SDS-PAGE, most of the fluorescent enzyme was located in the stacking gel, with a small amount entering the running gel as a series of faint bands, forming a ladder (not shown) with no fluorescent material at the 14-kDa region. These observations strongly suggest that the enzyme forms large aggregates in the presence of SDS.

Enzyme Reaction Products—To determine where in the LPXTG motif cleavage occurred, purified endopeptidase was used to cleave a bead-bound form of the KRQLPSTGETANPFY peptide, and the cleaved fragment was subjected to N-terminal sequence analysis. Results revealed that the enzyme cleaved after glutamic acid within the LPSTGE sequence, releasing the TANPFY peptide fragment (Fig. 4A). On the other hand, the enzyme cleaved the free KRQLPSTGETANPFY peptide at two sites, after the serine and the glutamic acid of LPSTGE, yielding TGETANPFY and TANPFY, respectively (Fig. 4B). The fact that only these products were observed indicates that the trypsin (KR) and chymotrypsin (FY) substrates found at either end of the peptide were not cleaved by the endopeptidase. When the enzyme was reacted with similar bead-bound peptides in which the LPSTGE was reversed (EGTSSL) or randomly placed (TEPGLS), no cleavage was observed (not shown). No cleavage was also observed when native bovine serum albumin was reacted in a similar way. The small amount of radioactive materials observed at the solvent front originated from the impurities in the synthetic peptide. These impurities were not reactive to enzyme action. Based on its ability to specifically cleave within the LPXTG anchor motif of surface proteins, we have termed this endopeptidase "LPXTGase."

Enzyme Kinetics—Since the enzyme does not contain any aromatic amino acid, neither the Lowry nor Bradford methods could be used to determine protein concentration. Thus, the enzyme concentration was determined on the basis of its estimated molecular mass (14 kDa) and dry weight. Using this, a Lineweaver-Burk plot of the kinetics of the LPXTGase was determined (Fig. 5), resulting in a K_m of 0.26 mM and a V_max of 67 μM in 30 min when 2.4 μM enzyme was used in the assay.

Optimal Conditions for Enzyme Activity—As shown in Fig. 6, the LPXTGase exhibited a broad pH optimum between 7.5 and 10. Below pH 5 or above 10, the enzyme activity could not be measured. In most enzyme assays, pH 7.6 was used, because background activity was higher at higher pH. Enzyme activity was highest when 0.1–0.2% of both Brij 35 and Triton X-100 was incorporated. The maximal initial enzyme velocity was maintained up to the first 20 min, and after 1 h only small additional cleavage of the peptide occurred.

Inhibition of Activity by Salts—The LPXTGase was exposed to a variety of salts, and its activity was tested for cleavage of the LPXTG-containing peptide. As seen in Table I, the enzyme was found to be rather sensitive to exposure to a number of certain salts.

Presence of Carbohydrate in the LPXTGase—Successful linking of 125I-labeled tyramine to the enzyme demonstrated that it possesses free carboxyl groups. Nonetheless, the enzyme did not bind to DEAE-cellulose, indicating that these carboxyl groups are not surface-exposed. It seemed plausible that the charged amino acids of the enzyme are internalized and that the hydrophobic amino acids are located on the exterior surface in view of the fact that the enzyme is likely to be associated with the cell membrane. However, an enzyme with a hydrophobic surface would not be very soluble in aqueous buffer, which is contrary to our findings. This suggested to us that a few residues of sugars, which would prevent surface exposure of the carboxyl groups but would confer surface hydrophilicity to the enzyme, might shield the carboxyl groups, allowing them to remain soluble in aqueous buffer. To test this, the enzyme was incubated with 5 mM of periodate in 20 mM phosphate buffer, pH 6, for 4 h at 4 °C, and enzyme activity was measured. We found that this treatment nearly completely abolished the enzyme activity, whereas periodate treatment of trypsin in an identical manner showed no effect on its enzyme activity.

When the sugar composition of the LPXTGase was analyzed, we found 1-fucose, D-galactose, D-galactosamine, D-glucose, D-glucosamine, and D-mannose in a molar ratio of 1:2:3:13:2:2 (Fig. 7). The aggregate mass of the oligosaccharide is 3,936
Inactivation of LPXTGase activity by glycosidases. A fixed amount of LPXTGase was preincubated for 1 h with varying amounts of glycosidases at 37 °C in a 40-μl reaction volume of 30 mM Tris-HCl, pH 7.6, containing 0.1% Brij 35. At this time, 10 μl of 125I-labeled, bead-bound LPXTG peptide substrate (about 180,000 cpm) was added, the mixture was incubated at 37 °C for an additional 1 h, and the radioactivity of the cleaved peptide fragment was determined. Triangle, β-galactosidase; square, N-acetylhexosaminidase; diamond, β-glucosidase.

The amino acid composition of LPXTGase was determined by the Rockefeller University Protein Chemistry Laboratory.

| Amino acids | Number of residues |
|-------------|-------------------|
| Asn/Asp     | 5                 |
| Gln/Glu     | 10                |
| Ser         | 3                 |
| Thr         | 1                 |
| Gly         | 5                 |
| Ala         | 24                |
| Pro         | 3                 |
| Val         | 1                 |
| Leu         | 1                 |
| Ile         | 1                 |
| Lys         | 7                 |
| Unknown     | ?                 |

The amino acid composition of LPXTGase was determined by the Rockefeller University Protein Chemistry Laboratory.
compound, with a retention time close to that for PTH-phenylalanine, the mass spectroscopy showed three distinct peaks with masses of 102.2, 288.1, and 519.2 Da, which do not correspond with those of known PTH-derivatives. Acid hydrolysis of the fourth PTH-derivative gave rise to identical results as the third PTH-derivative. Meanwhile, the acid hydrolysate of the fifth PTH-derivative contained two species with masses of 102.1 and 212.1, the latter representing unhydrolyzed material. Acid hydrolysate of the sixth PTH-derivative contained two species with masses of 102.1 and 288.0, the latter also representing the unhydrolyzed material. Thus, we could only conclude from the species generated by acid hydrolysis that the third, fourth, fifth, and sixth PTH-derivatives are not common amino acids.

DISCUSSION

The LPXTGase of *S. pyogenes* is an unusual enzyme in many respects. The enzyme is glycosylated, and the carbohydrate moiety appears to be essential for enzyme activity. This suggests that a certain spatial arrangement of carbohydrate and protein backbone is necessary for enzyme activity. As far as we know, no precedent for a glycosylated enzyme exists in prokaryotes. However, in eukaryotes, C1r and C1s, the subcomponents of the complement C1, and prekallikrein, a protease in the blood clotting cascade, are known to be glycosylated (62, 63). It is not known whether these carbohydrates play any role in the proteolytic reaction.

In the LPXTGase, the protein backbone is extremely hydrophobic, which may be attributed to the presence of uncommon amino acids. This is supported by the fact that the PTH-compounds of these unusual amino acids moved nearly to the solvent front with a running solvent mixture of *n*-butyl alcohol/hexane/acetic acid/water (40:40:9:1). Seven lysine residues were found in the enzyme, yet tryptic digestion of the enzyme yielded only two fragments, indicating that either several lysine residues were not accessible to trypsin or that some lysines were modified. It is tempting to speculate that in living bacteria, the hydrophobic protein backbone is embedded in the cell membrane and that the hydrophilic carbohydrate is localized within the hydrophilic peptidoglycan layer. The salt sensitivity of the LPXTGase activity probably reflects an intimate association of the enzyme with the hydrophobic environment of membrane.

Direct determination of the purity of the purified LPXTGase by standard procedures such as SDS-PAGE analysis was not possible because of its ability to form large aggregates in the presence of SDS, and as a result it is unable to enter the resolving gel as a distinct protein band and barely enters the 4% stacking gel. The protein does not contain any aromatic amino acids, which explains its inability to absorb UV at 280 nm, and does not stain with Coomassie Blue or silver. Even when over 200 µg of purified enzyme was subjected to SDS-PAGE, no protein band was detected after staining with Coomassie Blue or silver. Even when over 200 µg of purified enzyme was subjected to SDS-PAGE, no protein band was detected after staining with Coomassie Blue or silver, indicating that the purified enzyme sample did not contain any stainable protein contaminant. We considered that the observed LPXTGase activity might be attributed to a very small amount of contaminant rather than the glycoprotein that we have identified. However, if this were true, we must conclude that the putative contaminant with endopeptidase activity is also a glycoprotein, because removal of sugars from purified endopeptidase preparations always abolished the enzyme activity.

Over the course of these studies, several independently purified LPXTGase samples were analyzed for amino acid sequence. In each analysis, 200 µg to over 1 mg dry weight of the enzyme sample was used. Since as little as a 0.1–µg sample of a 10-kDa peptide is sufficient for sequencing by the automated sequencer, 0.01–0.05% of contaminating proteins would have been detected by the instrument. In fact, 10 of our most puri-
fied samples could not be sequenced, and no false sequence data could be obtained, further verifying the purity of the preparation.

In contrast to the sortase described by Schneewind et al. (55), which cleaves the LPXTG sequence after threonine, our LPXTGase cleaves the bead-bound KRQLPSTGETANPFY after glutamic acid and cleaves the free form of the same pentadecapeptide after both serine and glutamic acid. In contrast to sortase, our LPXTGase does not have cysteine or methionine and is totally insensitive to sulfhydryl reagents. Another difference between LPXTGase and sortase is that hydroxylamine inhibits the activity of our LPXTGase, whereas hydroxylamine stimulates the activity of sortase. The LPXTGase activity was reduced by more than 70% in the presence of 100 mM hydroxylamine, and it was completely abolished in the presence of 200 mM.

**FIG. 10.** Analysis of an unusual amino acid present in peptide fragment 1. The third PTH-derivative of tryptic fragment 1, exhibiting a mass of 537.0, was hydrolyzed for 22 h with 6 N HCl at 110 °C, and the hydrolysis products were examined with reverse phase chromatography (A) and mass spectroscopy (B).
Another clear distinction between sortase and LPXTGase lies in the substrate cleavage activities of the enzymes. The \( K_{\text{cat}} \) value of 0.26 mM for LPXTGase for the KRQLPSTGETANPFY peptide was comparable with the reported \( K_{\text{cat}} \) values of 0.21, 0.36–3.1, and 0.08–0.5 mM for human Lys-plasmin (64), bovine trypsin (65), and a protease from S. aureus, respectively (66), for synthetic peptides. The calculated turnover value, or \( K_{\text{cat}} \), of the LPXTGase is 0.016/s. In comparison, the reported \( K_{\text{cat}} \) values for bovine trypsin ranged from 0.003 to 1.35/s, depending on the synthetic substrate used (65). When compared with the calculated \( K_{\text{cat}} \) of sortase from S. aureus (56), the 0.016/s \( K_{\text{cat}} \) value of LPXTGase from S. pyogenes is at least 2 orders of magnitude higher. It is estimated that there are at least five different surface proteins on a given Gram-positive bacterium that are anchored through the LPXTG motif, and there are probably thousands of copies of these molecules expressed in each organism. Thus, in order to properly anchor all these molecules during the 30–40-min division time of the bacterial cell, an enzyme with a high \( K_{\text{cat}} \) would be necessary to successfully accomplish this.

Many peptide antibiotics produced by Bacillus sp. contain unusual amino acids, and these peptides are synthesized non-ribosomally on amino acid-activating polypeptidyl templates. The overrepetitiveness of alanine in the LPXTGase seems unusual; however, another characteristic of nonribosomally synthesized peptides is the overrepetition of some amino acids. For example, tyrocidine, a cyclic decapeptide antibiotic from Bacillus brevis, contains ornithine and three phenylalanines, two of which are in the \( \beta \)-configuration. The mechanism of nonribosomal peptide synthesis was most extensively investigated previously in the synthesis of tyrocidine (67–70). Recently, the genes encoding the polypeptides of tyrocidine synthesis have been cloned (71).

It is surprising that S. pyogenes produces a small molecule that inhibits the activity of LPXTGase. We speculate that such an inhibitor may have some important regulatory function in the living bacterial cell. This is supported by the fact that S. pyogenes grown in the presence of this inhibitor fails to display M protein and fibronectin-binding protein on the cell surface, suggesting that the function of this LPXTGase may be essential for cell surface expression of these proteins. Another important question is whether the LPXTGase and its inhibitor are present in other Gram-positive bacteria. When one assumes that surface proteins in most Gram-positive bacteria become anchored to cell wall peptidoglycan by a common mechanism, it is likely that the answer is yes. This has been verified from our recent finding that S. aureus also produces an enzyme strikingly similar to the LPXTGase of S. pyogenes. The S. aureus enzyme cleaves the LPXTG motif, has a similar molecular weight, and is glycosylated like the S. pyogenes enzyme.

In addition, we found a low molecular weight inhibitor from S. aureus that inhibits both the activity of this enzyme and the LPXTGase from S. pyogenes. An interesting possibility exists, therefore, that analogues of LPXTGase and sortase are present in all Gram-positive bacteria and that these two enzymes function together to accomplish the cleavage of the LPXTG motif of surface proteins and the ultimate anchoring of the cleaved protein to the cell wall. Precisely how these two enzymes accomplish this is currently being investigated.

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\[ ^{2} \text{Lee and V. A. Fischetti, manuscript in preparation.} \]

\[ ^{3} \text{Lee and V. A. Fischetti, unpublished data.} \]
50. Mazmanian, S. K., Liu, G., Ten-That, H., and Schneewind, O. (1999) Science 285, 760–762.
51. Pancholi, V., and Fischetti, V. A. (1988) J. Bacteriol. 170, 2618–2624.
52. Schneewind, O., Model, P., and Fischetti, V. A. (1992) Cell 70, 267–281.
53. Navarre, W. W., and Schneewind, O. (1999) Microbiol. Mol. Biol. Rev. 63, 174–229.
54. Pullen, M. J., Lam, A. C., Antonio, M., and Dunbar, K. (2001) Trends Microbiol. 9, 97–102.
55. Schneewind, O., Fowler, A., and Faull, K. F. (1995) Science 268, 103–106.
56. Ton-That, H., Liu, G., Mazmanian, S. K., Faull, K. F., and Schneewind, O. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12424–12429.
57. Ton-That, H., Mazmanian, S. K., Allsne, L., and Schneewind, O. (2001) J. Biol. Chem. 277, 7447–7452.
58. Mazmanian, S. K., Liu, G., Jensen, E. R., Lenoy, E., and Schneewind, O. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5510–5515.
59. Nelson, D., Loomis, L., and Fischetti, V. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4107–4112.
60. Fischetti, V. A., Gotschlich, E. C., and Bernheimer, A. W. (1971) J. Exp. Med. 133, 1105–1117.
61. Loeffler, J. M., Nelson, D., and Fischetti, V. A. (2001) Science 294, 2170–2172.
62. Sim, R. B. (1981) Methods Enzymol. 80, 26–42.
63. Heimark, R. L., and Davie, E. W. (1981) Methods Enzymol. 80, 157–172.
64. Robbins, K. C., Summara, L., and Wohl, R. C. (1981) Methods Enzymol. 80, 379–387.
65. Biswas, B., Adhya, S., Washart, P., Paul, B., Tröstel, A. N., Powell, B., Carlton, R., and Merril, C. R. (2002) Infect. Immun. 70, 204–210.
66. Drapeau, G. R. (1976) Methods Enzymol. 45, 469–475.
67. Lee, S. G., Roskoski, R., Jr., Bauer, K., and Lipmann, F. (1973) Biochemistry 12, 388–405.
68. Lee, S. G., and Lipmann, F. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 607–611.
69. Lee, S. G., and Lipmann, F. (1975) Methods Enzymol. 43, 585–602.
70. Lee, S. G., and Lipmann, F. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 2343–2347.
71. Mootz, H. D., and Marahiel, M. A. (1997) J. Bacteriol. 179, 6843–6850.
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