Multiple Enzymatic Activities of the Murein Hydrolase from Staphylococcal Phage φ11

IDENTIFICATION OF A D-ALANYL-GLYCINE ENDOPEPTIDASE ACTIVITY*

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Bacteriophage muralytic enzymes degrade the cell wall envelope of staphylococci to release phage particles from the bacterial cytoplasm. Murein hydrolases of staphylococcal phages φ11, 80a, 187, Twort, and φPVL harbor a central domain that displays sequence homology to known N-acetylmuramyl-l-alanyl amidasases; however, their precise cleavage sites on the staphylococcal peptidoglycan have thus far not been determined. Here we examined the properties of the φ11 enzyme to hydrolyze either the staphylococcal cell wall or purified cell wall anchor structures attached to surface protein. Our results show that the φ11 enzyme has D-alanyl-glycyl endopeptidase as well as N-acetylmuramyl-l-alanyl amidas activity. Analysis of a deletion mutant lacking the amidase-homologous sequence, φ11(Δ181–381), revealed that the D-alanyl-glycyl endopeptidase activity is contained within the N-terminal 180 amino acid residues of the polypeptide chain. Sequences similar to this N-terminal domain are found in the murein hydrolases of staphylococcal phages but not in those of phages that infect other Gram-positive bacteria such as Listeria or Bacillus.

The cell wall envelope of Gram-positive bacteria is a macro-molecular, exoskeletal organelle that is assembled and turned over at designated sites (1). The cell wall functions not only to protect bacteria from osmotic lysis but also serves as a surface organelle that allows Gram-positive pathogens to interact with their environment, most notably the infected tissues of the host (2). To understand the mode of action of antibiotics and to identify new targets of antibacterial therapy, Staphylococcus aureus has been employed as a model organism to study the physiology of the cell wall of Gram-positive bacteria for almost 50 years (3–5). The characterization of muralytic enzymes has been instrumental in understanding cell wall turnover as well as determining the structure of peptidoglycan (6).

The peptidoglycan of S. aureus consists of a repeating disaccharide, N-acetylmuramic acid-(β1–4)-N-acetylglycosaminoglycose (MurNAc-GlcNAc) (4, 7). The d-lactyl moiety of N-acetylmuramic acid is amide-linked to the short peptide component of peptidoglycan (8–10). Wall peptides are cross-linked with other peptides attached to neighboring glycan strands, thereby generating a three-dimensional network that surrounds the staphylococcal cell (11–13). During cell wall synthesis the peptidoglycan precursor molecule, lipid II (C55-PP-MurNAc-(l-Ala-d-iGln-l-Lys-(NH2-Gly)₃)-d-Ala-d-Ala-COOH)-GlcNAc; C55-PP is undecaprenyl pyrophosphate), is incorporated into the peptidoglycan network via transglycosylation and transpeptidation reactions (14–17). Whereas transglycosylation leads to the polymerization of the glycan strands, the transpeptidation reaction results in the cross-linking of the peptide backbone of the cell wall (18). During this reaction, the terminal d-Ala of the pentapeptide precursor (l-Ala-d-iGln-l-Lys-(NH2-Gly)₃)-d-Ala-d-Ala-COOH) is removed and the carboxyl of d-Ala at position four is linked to the free amino of the pentaglycine cross-bridge within cell wall peptides of neighboring peptidoglycan strands (19). Fig. 1 shows the structure of the staphylococcal peptidoglycan and the cleavage sites of muralytic enzymes.

Several investigators employed biochemical techniques to isolate and characterize staphylococcal murein hydrolases, leading to the identification of N-acetylmuramyl-l-alanine amidase, N-acetylglycosaminidase, d-Ala-Gly endopeptidase, as well as Gly-Gly endopeptidase activities (6, 20–23). The isolation of staphylococcal transposon variants that are defective in autolysis has permitted the assignment of specific functions to individual genes as well as sequence comparison between murein hydrolases from many different bacterial species (24, 25). Murein hydrolases can be grouped into two separate classes of enzymes. The first group is the autolysins, for example S. aureus N-acetylmuramyl-l-alanine amidase (Atl), N-acetylglycosaminidase (Atl) (24), as well as the presumed Gly-Gly endopeptidase (LytM) (26). Autolysins are secreted from the bacterial cytoplasm by N-terminal signal peptides and degrade the cell wall peptidoglycan at specific sites during physiological growth and/or during stationary phase. Bacteriophage-encoded murein hydrolases, also called endolysins, are exported by holins, small polypeptides that are inserted into

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the cytoplasmic membrane of the bacterial host (27). Endolysins function to completely hydrolyze the cell wall peptidoglycan as a means to release bacteriophage particles from the bacterial cytoplasm.

The gene encoding the murein hydrolase of staphylococcal phage \( \text{f}11 \) has been sequenced (25). Sequence comparison revealed that this endolysin has a modular organization consisting of an N-terminal, a central, and a C-terminal domain. The central domain of the \( \text{f}11 \) enzyme displays sequence similarity with known N-acetylmuramyl-l-alanine amidas (amidase), muramidase, glucosaminidase, and lysozyme. \( \text{B} \), schematic diagram of the murein hydrolases from staphylococcal phage \( \text{f}11 \) (GenPept accession number 113675 (25)), \( \text{80} \alpha \) (accession number 1763243 (64)), \( \text{Twort} \) (accession number 2764981 (43)), and \( \text{f}11 \) (accession number 2764983) (43). Also shown is a schematic diagram of the deletion construct \( \text{f}11(\text{A}181-301) \), generated by removal of the central amidase portion of the full-length \( \text{f}11 \) enzyme. \( \text{C} \), sequence alignment of the predicted N-terminal domains of the enzymes shown in \( \text{B} \). Optimal sequence alignment was carried out via the World Wide Web using the Multiple Sequence Alignment version 2.1 (66), provided by the Institute for Biomedical Computing at Washington University, St. Louis, MO.

We have used purified recombinant \( \text{f}11 \) murein hydrolase to solubilize staphylococcal surface proteins from the bacterial cell wall (36). When analyzed on SDS-PAGE, surface protein migrated as a spectrum of fragments on SDS-PAGE with increasing mass (35). This can be explained as surface protein linked to peptidoglycan with different amounts of cross-linked cell wall subunits.

**Experimental Procedures**

**Strains and Materials**—Staphylococcal strain OS2 has been previously described (31) and was used as a host for the recombinant plasmid pHIT4 encoding the recombinant surface protein Seb-MH,-Cws (36). *Escherichia coli* strain BL21(DE3) (38) was used as a host for the expression of the 6His-tagged \( \text{f}11 \) and \( \text{f}11(\text{A}181-381) \) enzymes. The coding sequence of the 6His-tagged \( \text{f}11 \) amida was contained on plasmid pHIT2 (36). The truncated mutant protein, \( \text{f}11(\text{A}181-381) \), was generated by the ligation of DNA fragments encoding the N-
terminal 180 amino acids and C-terminal 100 amino acids of the full-length φ11 protein. DNA encoding the C-terminal domain of the φ11 hydrolase was polymerase chain reaction-amplified from pHTT2 DNA using the primers LA-Kpn (28) and φ11-BamHIs (AAAGCCCTCCAGTGAATTCTGTTAGGTTGTGG). The resulting polymerase chain reaction products were digested with BamHI and KpnI and NdeI and KpnI and subsequently cloned in a three-way ligation into the NdeI site of the vector pET9a (38) to generate pWil54. Tryptic soy broth, and culture was chilled to 4 °C. Cells were harvested by centrifugation at 30,000 g for 5 min. Broken cell walls were collected by centrifugation at 30,000 × g for 15 min, and the pellet was suspended in 50 mM Tris-HCl, pH 7.5. Walls were treated with α-amylase (Sigma, 100 μg/ml), DNase (Sigma, 10 μg/ml), and RNase A (Qiagen, 50 μg/ml) for 2 h at 37 °C. CaCl2 was added to a final concentration of 10 mM, and protein was removed from the peptidoglycan by the addition of trypsin (Sigma, 100 μg/ml) and incubating the walls overnight at 37 °C. Walls were collected by centrifugation, washed twice with water, once with 8 M LiCl, once with 100 mM EDTA, twice more with water, and finally with acetone. Walls were suspended in water and stored at –20 °C.

Preparation of C-terminal Anchor Peptides—Preparation of C-terminal anchor peptides from Seb-MH-Cws was carried out as described previously (35). Purified Seb-MH-Cws was methanol/chloroform-precipitated, dried under vacuum in a Speed-Vac concentrator (Savant), re-dissolved in water, and dialyzed against 50 mM Tris-HCl, pH 7.5, and an additional 50 mM Tris-HCl, pH 7.5, was added. The mixture was incubated at 20 °C for 20 min. Enzymes were fast protein liquid chromatography purified from the supernatant over a 1-ml bed volume column of nickel nitrotriacetic acid resin (Qiagen). The column was pre-equilibrated with buffer Q1 (8 mM urea, 100 mM NaH2PO4, 0.01 mM Tris-HCl, pH 7.3) and, after loading with sample, was washed with an additional 10 ml of buffer Q1. The column was washed with 15 ml of 30% Q1 and 70% Q2 (8 mM urea, 100 mM NaH2PO4, 0.01 mM Tris-HCl, pH 4.2). Protein was eluted from the column in a pH-dependent manner by a step gradient to 100% Q2 buffer.

The purified enzymes, in approximately 5 ml of Q2 buffer, were folded by dialysis against 1 liter of dialysis buffer (50% glycerol, 50 mM sodium phosphate, 10 mM MgCl2, 2 mM diithiothreitol, pH 6.5) for 4–6 h at 4 °C without stirring and then washed with 10 ml of buffer Q1. The column was then washed with 15 ml of 30% Q1 and 70% Q2 (8 mM urea, 100 mM NaH2PO4, 0.01 mM Tris-HCl, pH 4.2). Protein was eluted from the column in a pH-dependent manner by a step gradient to 100% Q2 buffer.

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and suspended in 1–3 ml of 70% formic acid. Five mg of cyanogen bromide was added, and the cleavage reaction was incubated for 16–18 h at room temperature in the dark. The cleaved peptides were dried under vacuum, washed twice with water, and suspended in 1–2 ml of buffer A. Samples were loaded onto a column packed with 1 ml of nickel nitritotriacetic acid pre-equilibrated with 10 ml of buffer A. The Column was washed with 10 ml of each buffer A, buffer B (8 M urea, 100 mM NaCl to give an initial NaH2PO4 (pH 2.5) to 30% (v/v) methanol in 100 mM NaH2PO4 (pH 2.8) at 37 °C. Digestion was terminated by the addition of 20% phosphoric acid.

The purification and rpHPLC analysis of muropeptides generated by the digestion of intact peptidoglycan with the α11 and α11/Δ381–381 enzymes was carried out using highly purified peptidoglycan as a substrate. Approximately 2 mg of pure staphylococcal peptidoglycan (see above) was digested with approximately 400–500 μg of enzyme in 1 ml of 50 mM Tris-HCl, 100 mM NaCl, pH 7.5, for 4 h at 37 °C. Enzyme was precipitated by the addition of trifluoroacetic acid to 10% and centrifugated after 15 min on ice. Removal of linked teichoic acids from the soluble muropeptides was achieved by heating the soluble muropeptides to 60 °C for 14 h. Muropeptides were desalted over a C18 cartridge and dried. The muropeptides were resuspended and reduced with sodium borohydride as described for the anchor muropeptides.

Separation of muropeptides by rpHPLC was carried out using a C18 column and a method devised for E. coli wall peptides (40) that was later modified for the separation of S. aureus muropeptides (39). The C18 column employed for the separation of muropeptides from anchor peptides (2 × 250 mm, C18 Hypersil, Keystone Scientific) contained a slightly different resin than the C18 column employed for the separation of muropeptides from purified peptidoglycan (2 × 250 mm, C18 Aquasil, Keystone Scientific), resulting in a discrepancy in the retention times observed between the two experiments. A linear gradient was started immediately after injection of 5% (v/v) methanol in 100 mM NaH2PO4 (pH 2.5) to 30% (v/v) methanol in 100 mM NaH2PO4 (pH 2.8) in 100 min. Azide was not added to the start buffer, and base-line drift was accounted for by subtracting the chromatogram of a blank run. Eluted muropeptides were monitored at 206 nm, and positive fractions were desalted using a C18 cartridge and dried under vacuum prior to analysis by ESI-MS or Edman degradation.

**Chemical Analysis of Solubilized Muropeptides by Modified Morgan-Elson and DNFB Assays—**Crude staphylococcal cell walls were resuspended in water (20 mg wet weight/ml) to make a stock solution. Into each reaction tube, 400 μl of wall stock was mixed with 500 μl of 100 mM NaH2PO4, pH 7.5 (or pH 6.0 for mutanolysin reaction), and 100 μl of NaCl to give an initial A560 of 1.1. Lysostaphin (10 μg/ml), α11 enzyme (100 μg/ml), α11/Δ381–381 (100 μg/ml), or mutanolysin (50 units/ml)
monitoring of eight PPG solution signals (singly charged ions at m/z fragmentation ions indicated. B compound of bration across the to generate the color reagent. To the boiled muropeptides, 900 a stock reagent. 1 ml of stock reagent was diluted per 7 ml of acetic acid benzaldehyde (Ehrlich’s reagent) was dissolved into a total volume of p were mixed with 100 ethanol) was added, and the mixture was heated to 60 °C for 30 min. linked to wall peptides. hexosamines and those that exist as disaccharides or are amidically known discrepancies between the extinction coefficient of free GlcNAc was read spectrophotometrically at 585 nm, and samples were compared with standards of GlcNAc of known concentration. GlcNAc standards were eluted in K$_2$B$_4$O$_7$ for 7 min instead of 30 min to compensate known discrepancies between the extinction coefficient of free N-acetylhexasamines and those that exist as desaccharides or are amicidally linked to wall peptides.

The liberation of free amino groups was measured by mixing 100 μl of the soluble wall digestion products with 100 μl of 2% K$_2$B$_4$O$_7$ in water (22). To this, 20 μl of DNFB solution (130 μl of DNFB in 10 ml of ethanol) was added, and the mixture was heated to 60 °C for 30 min. The reaction was stopped by the addition of 800 μl of 2 N HCl, and absorbance was read at 420 nm. All experiments were performed in triplicate and compared with a standard of L-alanine.

ESI-MS of Muropeptides—Dried muropeptides were dissolved in 30 μl of water:CH$_3$CN:formic acid (50:50:0.1). A Perkin-Elmer Sciex API III triple quadruple mass spectrometer was tuned and calibrated by flow injection (10 μl/min) of a mixture of PPG 425, 1000, and 2000 (3.3 × 10$^{-8}$, 1 × 10$^{-4}$, and 2 × 10$^{-4}$ m, respectively) in water:methanol (1:1) containing 2 mM ammonium formamide and 0.1% CH$_3$CN. Calibration across the m/z range 10–2400 was achieved by multiple ion monitoring of eight PPG solution signals (singly charged ions at m/z 55.99, 326.25, 906.67, 1254.92, 1545.13, 1863.34, and 2010.47 and the doubly charged ion at m/z 520.4). The ion spray voltage was operated at 4.5 kV using hydrocarbon-depleted air for the spray nebulization, and spectra were generated with a curtain gas produced from the vapors of liquid nitrogen. Samples were introduced into the ionization source by flow injection. ESI-MS spectra were obtained at instrument conditions sufficient to resolve the isotopes of the PPG/NH$_3^+$ singly charged ion at m/z 906 with 40% valley, an orifice voltage of 60, and step size during data collection of 0.3 Da. Daughter ion spectra were obtained using degraded mass resolution to improve sensitivity of detection, and a step size of 1 Da was used for data collection. Under these conditions, the isotopes of the PPG/NH$_3^+$ singly charged ion at m/z 906 were not resolved from one another.

RESULTS

φ11 Murein Hydrolase Digestion of Cell Wall Anchor Structures from Staphylococcal Surface Proteins—Previous characterization of φ11 murein hydrolase-released cell wall anchor structures of surface proteins revealed the presence of linked cell wall tetrapeptide (L-Ala-D-iGln-L-Lys-(surface protein-Gly$_5$)-D-Ala-COOH) and disaccharide-tetrapeptide (MurNAc-(L-Ala-D-iGln-L-Lys-(surface protein-Gly$_5$)-D-Ala-COOH)-GlcNAc) (36). This observation suggested that the φ11 murein hydrolase may cleave the peptidoglycan at two sites, the amide bond between N-acetylmuramyl and L-Ala and the peptide bond between β-Ala and Gly. If so, φ11 murein hydrolase digestion of muramidase-released surface protein should yield L-Ala-D-iGln-L-Lys-(surface protein-Gly$_5$)-D-Ala-COOH. Indeed, the mobility of the doubly digested surface protein on SDS-PAGE was found to be identical to surface protein solubilized directly from the staphylococcal peptidoglycan with the φ11 enzyme (Fig. 2C), indicating that the attached peptidoglycan subunits had been removed.

To examine the structures of the muropeptides removed from the surface protein by the φ11 enzyme, we employed a strategy previously devised to analyze the cell wall anchor structures of staphylococcal surface proteins (35, 36). Seb-MH$_6$-Cws is a fusion between staphylococcal enterotoxin B and the cell wall sorting signal of protein A (Fig. 2A). At the fusion site between these domains a methionine followed by six histidines has been inserted. After solubilization of the staphylococcal cell wall with the muramidase mutanolysin, Seb-MH$_6$-Cws was purified by affinity chromatography on nickel resin and cleaved by cyanoan bromide close to the anchoring point of the polypeptide with the cell wall. The C-terminal anchor peptides were purified by a second round of affinity chromatography and were then digested with φ11 murein hydrolase. When separated by rpHPLC, the doubly digested cell wall anchor peptides generated three major and several minor peaks of absorption at 206 nm (Fig. 2B). The major peaks (designated A, B, and C) were desalted, concentrated, and analyzed by ESI-MS.

The sample that eluted at 7.2% methanol (peak A) generated an ion with m/z 702.4, a measurement that is in close agreement with the structure of a peptidoglycan cleavage fragment NH$_2$-t-Ala-d-iGln-l-Lys-(NH$_2$Gly$_5$)-d-Ala-COOH (calculated ion mass (MH$^+$) 702.74). The sample that eluted at 9.9% methanol (peak B) yielded two ions with m/z 1182.8 and 979.7, observations that correspond to the peptidoglycan structures MurNAc-t-Ala-d-iGln-l-Lys-(NH$_2$Gly$_5$)-d-Ala-COOH (calculated MH$^+$ 1182.21) and MurNAc-t-Ala-d-iGln-l-Lys-(L-$N$-$N$-Gly$_2$Gly$_5$)-d-Ala-COOH (calculated MH$^+$ 979.02), respectively. As can be observed from the chromatogram, peak B was eluted near the expected time of MH$^+$ 1182.21. As can be observed from the chromatogram, peak B was eluted near the expected time of MH$^+$ 1182.21. The fusion site between these domains a methionine followed by six histidines has been inserted. After solubilization of the staphylococcal cell wall with the muramidase mutanolysin, Seb-MH$_6$-Cws was purified by affinity chromatography on nickel resin and cleaved by cyanogen bromide close to the anchoring point of the polypeptide with the cell wall. The C-terminal anchor peptides were purified by a second round of affinity chromatography and were then digested with φ11 murein hydrolase. When separated by rpHPLC, the doubly digested cell wall anchor peptides generated three major and several minor peaks of absorption at 206 nm (Fig. 2B). The major peaks (designated A, B, and C) were desalted, concentrated, and analyzed by ESI-MS.

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Compounds eluted after HPLC chromatography as shown in Fig. 2 were desalted and analyzed by ESI-MS or subjected to protein sequence analysis. No amino acids other than glycine and alanine were detected in significant amounts during the sequencing reaction. Numbers in parentheses indicate the concentration of cleaved phenylthiohydantoin residues in picomoles.

These ESI-MS data further corroborated our hypothesis that the \( \phi 11 \) hydrolase may cut muramidase-released cell wall an-chor structures at two positions, the amide bond between \( N \)-acetylmuramyl and \( \alpha \)-Ala and the peptide bond between \( \alpha \)-Ala and Gly. Nevertheless, the ESI-MS data cannot distinguish between \( \alpha \)-Ala-Gly or Gly-Gly endopeptidase activity, as the compound mass of 702.74 could be explained as peptides with the structure \( \text{NH}_2-\text{Ala-}d-i\text{Gln-}l-Lys-(\text{NH}_2-\text{Gly}_3)-d-\text{Ala-COOH} \), \( \text{NH}_2-\text{Ala-}d-i\text{Gln-}l-Lys-d-\text{Ala-Gly}_2\text{-COOH} \), or any permutation thereof. To distinguish between these possibilities we examined the structure of the ion with \( m/z \) 702.4 by CID in an MS/MS experiment (Table I and Fig. 3). We observed a fragment product ion at \( m/z \) 286, corresponding to an intact pentaglycine cross-bridge. We also observed a daughter ion of mass 614, a product of removal of a single \( d \)-alanine from position 4 of the stem peptide, indicating that the \( d \)-Ala at position four of the wall peptide was not substituted and that the pentaglycine was linked to the \( \epsilon \)-amino of lysine. See Table I for a listing of the observed daughter ions and an interpretation of their structure.

We would also expect that Edman degradation of the sample in peak A and peak B should yield five consecutive cycles of phenylthiohydantoin glycine if the \( \phi 11 \) enzyme displayed \( \alpha \)-Ala-Gly endopeptidase activity. This test was, and the concentration of phenylthiohydantoin glycine obtained after Edman degradation remained similar during five consecutive cleavage cycles. Equimolar amounts of phenylthiohydantoin alanine and glycine were released in cycle 1 of the peak A sample, whereas no such release of alanine was observed for material that eluted in peak B (Table II). This observation is consistent with the presence of an N-terminal alanine in the peak A but not in the peak B sample.

The ESI-MS, CID-MS/MS, and Edman degradation data indicate that peak A contained a branched peptide with the structure \( \text{NH}_2-\text{Ala-}d-i\text{Gln-}l-Lys-\text{(NH}_2\text{-Gly}_3)-d-\text{Ala-COOH} \) and with two N termini (Ala and Gly), whereas peak B contained a branched peptide with the structure \( \text{MurNAC-}l-\text{Ala-}d-i\text{Gln-}l-\text{Lys-}(\text{NH}_2\text{-Gly}_3)-d-\text{Ala-COOH} \) and only one N terminus (Gly). These data suggest that the \( \phi 11 \) murein hydrolase cut muramidase-released anchor peptides at two sites, the \( d \)-Ala-Gly peptide bond and the \( N \)-acetylmuramyl-\( l \)-Ala amide bond. Cleavage at the latter site appeared to be less effective as more than half of all cleavage products still contained linked disaccharide. This observation is similar to the direct solubilization of surface protein with \( \phi 11 \) murein hydrolase, which yielded equal amounts of anchor peptides with linked tetrapeptide (\( \text{NH}_2-\text{Ala-}d-i\text{Gln-}l-\text{Lys-}(\text{surface protein-Gly}_5)-d-\text{Ala-COOH} \)) and disaccharide tetrapeptide MurNAC-\( l-\text{Ala-}d-i\text{Gln-}l-\text{Lys-}(\text{surface protein-Gly}_5)-d-\text{Ala-COOH} \).
tested the staphylocytic activity of the \( \phi 11(\Delta 181–301) \) enzyme in the presence of protease inhibitors, and we found that the activity could be completely abolished in the presence of 1 mM p-hydroxymercuribenzoic acid, an organic mercurial known to form stable complexes with thiol moieties (cysteine) (Fig. 4) (41). The purified enzyme was partially inhibited in the presence of 5 mM EDTA; however, a mixture of pepstatin and PMSF, inhibitors of aspartate and serine proteases, had no effect on the cell wall hydrolis activity of the \( \phi 11(\Delta 181–301) \) enzyme. These data suggest that the conserved cysteine residue is required for the \( \nu \)-Ala-Gly endopeptidase activity of the N-terminal portion of \( \phi 11 \) murein hydrolase.

Many murataic enzymes possess glycan hydrolase activity as indicated by the release of reducing \( N \)-acetylhexosamines (22). We analyzed the \( \phi 11 \) and \( \phi 11(\Delta 181–301) \) enzymes for such an activity against crude staphylococcal cell walls using a modified Morgan-Elson procedure. As indicated in Table III, neither the \( \phi 11 \) nor \( \phi 11(\Delta 181–301) \) enzyme released significant hexosamine from staphylococcal cell walls. As a control, mutanolysin, an \( N \)-acetylmuramidase, released reducing hexosamines, whereas lysostaphin, a glycyl-glycine endopeptidase, did not. We measured the activity of murein hydrolases to release free amino groups from the staphylococcal cell wall with the Sanger reagent (DNFB) and measuring the absorbance of each sample compared to a set of \( L \)-alanine standards of known concentration (22).

### Table III

| Enzyme          | Absorbance, 585 nm | \([N\text{-Acetylhexosamines}]^a\) | Absorbance, 420 nm | \([\text{Free NH}_2\text{ groups}]^b\) |
|-----------------|-------------------|-----------------------------------|-------------------|-----------------------------------|
| Lysostaphin     | 0.0000 ± 0.0002   | Not detectable                    | 0.8420 ± 0.0251   | 2.691 ± 0.080                     |
| Mutanolysin     | 0.4689 ± 0.0069   | 0.637 ± 0.009 mM                  | 0.1185 ± 0.0442   | 0.202 ± 0.075                     |
| \( \phi 11 \)   | 0.0002 ± 0.0001   | Not detectable                    | 0.7868 ± 0.0457   | 2.501 ± 0.145                     |
| \( \phi 11(\Delta 181–301) \) | 0.0018 ± 0.0069   | 0.020 ± 0.003 mM                  | 0.6287 ± 0.0387   | 1.957 ± 0.121                     |

\( ^a \) The concentration of \( N \)-acetylhexosamines was determined with the modified Morgan-Elson reaction (22) by measuring the absorbance at 585 nm and comparing with a set of GlcNAc standards of known concentration. As differences between the extinction coefficients of free (unsubstituted) hexosamines and those substituted with wall peptides have been observed (22), we have attempted to reduce the discrepancy by boiling the standards in borate buffer for 7 min instead of 30 min for the solubilized muropeptides (see “Experimental Procedures” for a detailed protocol).

\( ^b \) The concentration of amino groups was determined by modification with the Sanger reagent (DNFB) and measuring the absorbance of each sample compared to a set of \( L \)-alanine standards of known concentration (22).

### Table IV

**Edman degradation of muropeptides generated by the digestion of purified staphylococcal peptidoglycan**

Edman degradation of muropeptides generated from \( \phi 11 \) and \( \phi 11(\Delta 181–301) \) digestion of purified staphylococcal cell walls (pmol) released per cycle indicated in parentheses. Glycine and alanine were the only phenylthiohydantoin amino acids observed in significant amounts during sequencing.

| Enzyme           | Cycle 1   | Cycle 2   | Cycle 3   | Cycle 4   | Cycle 5   |
|------------------|-----------|-----------|-----------|-----------|-----------|
| \( \phi 11 \)    | Gly (640) | Gly (323) | Gly (194) | Gly (132) | Gly (92)  |
|                  | Ala (450) | Ala (189) | Ala (127) | Ala (100) | Ala (68)  |
| \( \phi 11(\Delta 181–301) \) | Gly (815) | Gly (603) | Gly (533) | Gly (440) | Gly (383) |
|                  | Ala (86)  | Ala (55)  | Ala (52)  | Ala (45)  | Ala (41)  |

**Purification of Peptidoglycan Cleavage Products of the \( \phi 11 \) Enzymes—**To analyze the digestion products of the \( \phi 11 \) or \( \phi 11(\Delta 181–301) \) enzymes by rpHPLC, digested staphylococcal peptidoglycan was further degraded with mutanolysin to cleave the glycan strands and liberate muropeptide monomers. After reduction of muropeptides with sodium borohydride, the sample was subjected to rpHPLC using the same procedure employed for the separation of muropeptides removed from the staphylococcal surface protein. The \( \phi 11 \) hydrolase-digested sample yielded one major peak that eluted at 5.6% methanol (Fig. 5A). Analysis of this sample by ESI-MS revealed a compound with \( m/z \) 702.3, corresponding to the mass of a singly charged muropeptide of the structure NH\(_2\)-t-Ala-\( \beta \)-Gln-L-Lys-(NH\(_2\)-Gly\(_2\)-t-Ala-COOH) (predicted mass 702.7). rpHPLC analysis of the \( \phi 11(\Delta 181–301) \) enzyme-digested sample also generated one major peak that eluted at 8.0% methanol (Fig. 5B). ESI-MS of the eluted sample revealed a single ion of \( m/z \) 1182.6. This measurement is in agreement with the predicted \( m/z \) of a singly charged muropeptide of the structure MurNAc-(\( \nu \)-Ala-\( \beta \)-Gln-L-Lys-(NH\(_2\)-Gly\(_2\)-t-Ala-COOH)-(\( \beta \)-1–4)-GlcNAc (calculated ion mass 1182.2). Taken together the data indicate that the \( \phi 11 \) murein hydrolase has \( N \)-acytulamuramyll-\( \nu \)-Ala amidase as well as \( \nu \)-Ala-Gly endopeptidase activity, whereas the \( \phi 11(\Delta 181–301) \) enzyme displays only \( \nu \)-Ala-Gly endopeptidase activity.

**Solubilization of Surface Protein with the \( \phi 11(\Delta 181–301) \) Enzyme—**If the \( \phi 11(\Delta 181–301) \) enzyme functions as a \( \nu \)-Ala-Gly endopeptidase, digestion of the staphylococcal cell wall with this enzyme should solubilize surface proteins linked to multiple muropeptide subunits that are attached along a single glycan strand. This was tested and \( \phi 11(\Delta 181–301) \)-solubilized Seb-MH\(_6\)-Cws was purified and subjected to SDS-PAGE. In
Staphylytic d-Alanyl-Glycine Endopeptidase Activity

The ion with m/z 2235 corresponds to anchor peptide linked to a wall subunit of the structure NH$_2$-D-Ala-d-iGln-D-Lys-(NH$_2$-H$_2$AQLPETF-Gly$_2$)-d-Ala-COOH, i.e. a single peptidoglycan subunit lacking the MurNAc-GlcNAc disaccharide (calculated MH$^+$ 2235). Ion groups centered at m/z 3238, 4439, 5631, and 6834 can be explained as anchor peptides attached to two, three, four, or five wall subunits where one of the subunits lacks the peptide substituent (a loss of 683.72 Da). Apparently, each of these cell wall structures was generated by N-acetylmuramyl-d-Ala amidase hydrolysis of one of the murein subunits. We speculate that these compounds are generated by the endogenous autolysin amidase activity in crudely prepared staphylococcal cell walls from which the Seb-MH$_6$-Cws protein was purified (42). Ions representing anchor peptides linked to multiple cell wall pentapeptides were not readily observed (usually the anchor peptides were linked to either a single or no pentapeptides). The reasons for this are most likely due to the fact that the staphylococcal peptidoglycan is highly cross-linked by transeptidation with the majority of subunits existing as tetrapeptides.

**DISCUSSION**

In this paper we analyzed the enzymatic properties of $\phi$11 murein hydrolase using both staphylococcal peptidoglycan as well as solubilized surface protein as substrates. We conclude that the enzyme is bifunctional, having both an N-acetylmuramyl-d-alanyl amidase and a d-alanyl-glycine endopeptidase activity. By engineering a deletion that removed the central amidase domain of this enzyme, we determined that the endopeptidase activity is contained within the first 180 residues of the polypeptide chain. To our knowledge this is the first report of such an activity associated with the aureophage endolysins. The presence of a muralytic activity in the N-terminal portion of the enzyme is in agreement with a recent report for the related muralytic enzyme of staphylococcal phage Twort (43). Like the $\phi$11 enzyme, the Twort murein hydrolase was characterized as an amidase based on the ability of the full-length enzyme to liberate DNFB reactive L-alanine from digested peptidoglycan (43). Because the N-terminal domain of the Twort enzyme is similar to that of the $\phi$11 hydrolase, we think it is likely that the Twort enzyme as well as other aureophages also display d-alanyl-glycine endopeptidase activity.

Recently it has been reported that the $\alpha$- and $\beta$-lytic proteases secreted by *Achromobacter lyticus* are capable of cleaving staphylococcal peptidoglycan at the pentaglycine cross-bridge (44–46). Both lytic proteases appear to hydrolyze not only the Gly-Gly bond but also d-Ala-Gly and Ala-Ala bonds, suggesting that these enzymes display a more relaxed substrate specificity than the $\phi$11 enzyme. The $\alpha$-lytic protease also possesses amidase activity indicating that it may be functionally similar to the $\phi$11 hydrolase, although the gene for this enzyme has not yet been cloned (45). The $\beta$-lytic protease is a metalloenzyme that bears no primary sequence homology to the $\phi$11 enzyme (44, 46).

Crude enzymatic preparations capable of cleaving the staphylococcal wall peptide-cross-bridge junction were also isolated over 30 years ago from *Streptomyces albus G* (47) and *Myxobacter AL-1* (10) culture filtrates. The contrast to surface protein released with the full-length $\phi$11 enzyme, the $\phi$11$(\Delta 181-301)$-solubilized species migrated as a spectrum of fragments on SDS-PAGE, similar to mutanolysin and amidase-released surface protein. Redigestion of the $\phi$11$(\Delta 181-301)$ solubilized surface protein with mutanolysin caused Seb-MH$_6$-Cws to migrate uniformly on SDS-PAGE, suggesting that the heterogeneity in mass is caused by linked murein subunits that are tethered along glycan strands (Fig. 6).

To examine the structure of these surface proteins further, the C-terminal anchor peptides of $\phi$11$(\Delta 181-301)$ solubilized Seb-MH$_6$-Cws were obtained by cyanogen bromide cleavage and affinity purification. MALDI-MS revealed the presence of a heterogeneous population of ions spaced at regular intervals (Fig. 6). The ion signal at m/z 2757 likely corresponds to anchor peptide linked to a single wall tetrapeptide of the structure MurNAc-\(\text{L-Ala-d-iGln-L-Lys(Gly}_2\text{-D-Ala)}(\text{1-4})\text{-GlcNAc}\) that is acetylated at the O-6 position of the muramic acid (calculated ion mass 2756.82). Ion groups centered at m/z 3019, 5152, and 6014 can be explained as anchor peptides linked to two, three, four, or five peptidoglycan subunits. A summary of the ions observed in each of these groups is given in Table V. The ion signals became more broadly dispersed with increasing mass and frequently diminished in intensity. These phenomena are likely due to the fact that the linked peptidoglycan subunits exist as either tetrapeptide (1162.16 Da) or pentapeptide subunits (1233.24 Da) that may or may not be acetylated at the O-6 position of N-acetylmuramic acid (42.03 Da). Thus, each additional linked peptidoglycan subunit increases the number of possible compounds by a factor of 4. Surface protein linked to three peptidoglycan subunits could theoretically exist as any one of 16 possible mass combinations, whereas a protein linked to four peptidoglycan subunits yields 25 different compounds. Hence a majority of the mass signals at m/z greater than 7000 overlapped and were difficult to interpret. Nonetheless, we were able to observe ion groups to an m/z of approximately 18,000, corresponding to anchor peptides linked to as many as 15 subunits (data not shown).

The ion with m/z 2235 corresponds to anchor peptide linked to a wall subunit of the structure NH$_2$-L-Ala-D-iGln-L-Lys-(NH$_2$-H$_2$AQLPETF-Gly$_2$)-d-Ala-COOH, i.e. a single peptidoglycan subunit lacking the MurNAc-GlcNAc disaccharide (calculated MH$^+$ 2235). Ion groups centered at m/z 3238, 4439, 5631, and 6834 can be explained as anchor peptides attached to two, three, four, or five wall subunits where one of the subunits lacks the peptide substituent (a loss of 683.72 Da). Apparently, each of these cell wall structures was generated by N-acetylmuramyl-d-Ala amidase hydrolysis of one of the murein subunits. We speculate that these compounds are generated by the endogenous autolysin amidase activity in crudely prepared staphylococcal cell walls from which the Seb-MH$_6$-Cws protein was purified (42). Ions representing anchor peptides linked to multiple cell wall pentapeptides were not readily observed (usually the anchor peptides were linked to either a single or no pentapeptides). The reasons for this are most likely due to the fact that the staphylococcal peptidoglycan is highly cross-linked by transeptidation with the majority of subunits existing as tetrapeptides.

**FIG. 5.** Comparison of muropeptides generated by digestion of purified staphylococcal peptidoglycan with the full-length $\phi$11 and $\phi$11$(\Delta 181-301)$ enzymes by rpHPLC. A, staphylococcal peptidoglycan was solubilized with the $\phi$11 murein hydrolase, and glycan strands were degraded by subsequent digestion of the solubilized muropeptides with mutanolysin (see “Experimental Procedures”). Digestion products were reduced with sodium borohydride and analyzed by rpHPLC on a C18 column using a protocol developed for the separation of staphylococcal muropeptides (39). Products eluting with the indicated absorption peak were desalted and analyzed by ESI-MS. The observed m/z of the ion signal and the proposed structure is shown. **B**, analysis of products generated by the solubilization of staphylococcal peptidoglycan with purified $\phi$11$(\Delta 181-301)$ enzyme.

![Comparison of muropeptides generated by digestion of purified staphylococcal peptidoglycan with the full-length $\phi$11 and $\phi$11$(\Delta 181-301)$ enzymes by rpHPLC.](http://www.jbc.org/Downloaded from http://www.jbc.org/ by guest on July 26, 2018)
glycan strand to which the anchor peptide is attached. Ions indicated in
by MALDI-MS. Ions indicated by * are apparently the result of partial amidase removal of peptide substituents from subunits along the linear
cyanogen bromide, affinity purification, and rpHPLC (see "Experimental Procedures"). HPLC fractions containing anchor peptides were analyzed
Inset near ion signals (see text).

Myxobacter enzyme was reported to have an additional, less
efficient amidase activity, which may indicate that it is also
functionally similar to the δ11 hydrolase (10). Staphylococcal
peptidoglycan is highly cross-linked (8, 11, 39, 48), and disruption
of the cross-bridges appears to be a highly efficient means
by which to rapidly hydrolyze the cell wall. It is likely not a
coincidence that enzymes whose function is to destroy the
murein sacculus, for example lysostaphin and phage hydro-
dases, have chosen the staphylococcal cross-bridge as their
target.

All aureophages described to date belong to the order of
tailed phages (caudovirales) and are further subclassified into
three families as follows: the Myoviridae, the Siphoviridae, or
the Podoviridae. Whereas δ187, δ11, and 80c belong to the
Siphoviridae family, Twort belongs to the Myoviridae (49).
That each of these phages encodes proteins homologous to the
D-Ala-Gly endopeptidase of δ11 suggests that this activity will
be found in several distantly related aureophages. Genes en-
coding phage hydrolases have been cloned and sequenced from
many different tailed phages that infect a variety of Gram-
positive bacteria including Listeria sp. (50), Streptococcus
pneumoniae (51–53), and Bacillus cereus (54). A comparison of
the muralytic enzymes from Gram-positive bacteria and their
phages has indicated that these enzymes are modular, contain-
ing separate catalytic and cell wall binding domains (targeting
domains) (51, 54, 55). It has been proposed that the evolution of
these enzymes has likely occurred through the swapping of
these domains and that phage lytic enzymes have co-evolved
with host autolysins (52, 56, 57). A majority of the muralytic
enzymes cloned from the Gram-positive phages are amidases
and bear significant homology to the central amidase portion of
the δ11 enzyme. Exceptions include the lytic enzymes of
phages A118 (Ply118) and A500 (Ply500) of Listeria monocytogenes,
which possess an L-alanyl-l-glutamate peptidase activity
(50), and the Cly glycosidase of the pneumococcal phage
Cp-1 (51). Due to the near ubiquitous presence of MurNAc-l-
alanine linkages in bacterial peptidoglycan, amidases fre-
quently display activity against cell walls from a wide variety

**Table V**

Summary of ions observed by MALDI-MS of δ11(Δ181–381)-solubilized anchor peptides

| Murein subunit | Wall peptides | 0 O-6 acetyl | 1 O-6 acetyl | 2 O-6 acetyl | 3 O-6 acetyl | 4 O-6 acetyl |
|---------------|---------------|-------------|-------------|-------------|-------------|-------------|
| Monomer       | 1 tetrapeptide | 2715.69     | 2775.19     |             |             |             |
| Monomer       | 1 pentapeptide| 2784.98     | 2827.91     |             |             |             |
| Dimer         | 2 tetrapeptides| 3876.21     | 3918.51     | 3962.02     |             |             |
| Dimer         | 1 tetrapeptide | 3947.16     | 3989.91     | ND          |             |             |
| Trimer        | 3 tetrapeptides| 5039.12     | 5081.15     | (5123.18)   | (5165.21)   | (5165.13)   |
| Trimer        | 2 tetrapeptides| (5093.12)   | (5081.15)   | (5123.18)   | (5165.21)   |             |
| Tetramer      | 4 tetrapeptides| (6201.28)   | (6243.31)   | (6285.34)   | (6327.37)   | (6370.19)   |
| Tetramer      | 3 tetrapeptides| (6272.36)   | (6314.03)   | (6356.35)   | (6399.56)   | (6440.48)   |
| Tetramer      | 1 pentapeptide | (6272.36)   | (6314.03)   | (6356.35)   | (6399.56)   | (6440.48)   |

**Fig. 6. Characterization of δ11 (Δ181–301)-solubilized anchor peptides by MALDI-MS.** Seb-MH 1-Cws was solubilized from the staph-
ylococcal cell wall with the δ11(Δ181–301) enzyme, and C-terminal anchor peptides were prepared by treatment of the full-length protein with
cyanogen bromide, affinity purification, and rpHPLC (see "Experimental Procedures"). HPLC fractions containing anchor peptides were analyzed
by MALDI-MS. Ions indicated by * are apparently the result of partial amidase removal of peptide substituents from subunits along the linear
glycan strand to which the anchor peptide is attached. Ions indicated in parentheses are approximate values due to a high degree of overlap with
nearby ion signals (see text). Inset near ion signals (see text).
of bacterial species. In contrast, we suspect that the \(\alpha\)11 enzyme may be specific toward only a small subset of the known types of peptidoglycan. Although \(\alpha\)-alanine-glycyl bonds have also been found in the peptidoglycan of strains within the genus \textit{Micrococcus}, \textit{Thermus} (58), and \textit{Deinococcus} (60), sequences homologous to the N-terminal domain of the \(\alpha\)11 hydrolase have thus far only been identified in enzymes isolated from staphylococci or their phages.

The C-terminal domain of the \(\alpha\)11 hydrolase bears significant homology to the wall targeting domain of \textit{lysostaphin} and \textit{InlB}, a protein found on the surface of \textit{L. monocytogenes} (61). Targeting domains have been shown to help control both the specificity and the level of activity of mureinolytic enzymes (28). Thus far, at least two other types of targeting domains have been characterized in autolysins of \textit{Gram-positive} bacteria. The first type to be identified was found in a set of bacterial and \(\alpha\) hydrolase have thus far only been identified in enzymes isolated from staphylococci or their phages.

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Multiple Enzymatic Activities of the Murein Hydrolase from Staphylococcal Phage φ 11: IDENTIFICATION OF A d-ALANYL-GLYCINE ENDOPEPTIDASE ACTIVITY

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