Substituents on the α-Carboxyl Group of D-Glutamic Acid in the Peptidoglycan of Several Bacterial Cell Walls*

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ABSTRACT: Several different carbohydrate-free peptides have been obtained from cell walls of *Staphylococcus aureus* and *Arthrobacter crystallopoietes* by degradation with lytic enzymes. They all contained one residue of amide ammonia per repeating subunit. A peptide obtained from cell walls of *Micrococcus lysodeikticus* contained no ammonia but had a glycine residue with a free carboxyl group.

It has been demonstrated by Edman degradation that both the amide ammonia in the former two cell walls and the COOH-terminal glycine in the latter are substituted on the α-carboxyl group of glutamic acid. Other features of the structures of these peptides are discussed.

The peptidoglycans of the cell walls of most, if not all, bacteria contain a polysaccharide (glycan) and a polypeptide. In the present paper one feature of this structure in three organisms, *Staphylococcus aureus*, *Arthrobacter crystallopoietes*, and *Micrococcus lysodeikticus*, will be considered. The glycans of these three organisms, in so far as they have been studied, seem to be similar or identical in structure, containing alternating β-1,4-linked residues of acetylglucosamine and acetylmuramic acid. Each acetylmuramic acid residue in the *S. aureus* peptidoglycan is substituted by a peptide which is cross-linked by pentaglycine cross bridges to form a polypeptide (Mandelstam and Strominger, 1961; Ghuysen *et al.*, 1965a; Petit *et al.*, 1966). The majority of the acetylmuramic acid residues in the *A. crystallopoietes* peptidoglycan are also substituted by a polypeptide which is shown in this and an accompanying paper (Tipper *et al.*, 1967) to contain a different kind of cross bridge. In *M. lysodeikticus*, however, the extent of peptide substitution is much less (Salton and Ghuysen, 1960; Leyh-Bouille *et al.*, 1966; Muñoz *et al.*, 1966a; Katz and Strominger, 1967). Other analytical data also indicate that there is considerable variation in the structure of the polypeptides, and it is on these differences that the present paper will focus.

Lysis of *S. aureus* cell walls with the *Chalaropsis* B endo-*N*-acetylmuramidase degrades the peptidoglycan into a soluble glycopeptide in which disaccharides are interlinked by the polypeptide. Peptidases isolated from *Streptomyces albus* G hydrolyze this glycopeptide within the polyglycine bridges, and the fragments produced have been shown to contain a tetrapeptide, L-alanyl-D-glutamyl-L-lysyl-D-alanine, linked at its amino-terminal end to acetylmuramic acid, and carrying the polyglycine component on the ε-amino group of L-lysine (Ghuysen *et al.*, 1965a). The amino terminus of the pentaglycine cross bridge could have been linked in the polypeptide to either of the potentially free carboxyl groups of the tetrapeptide, the COOH group of its D-alanine terminus, or the α-COOH group of D-glutamic acid (since the γ-COOH group is presumably linked to L-lysine, see below).

The tetrapeptide in this structure is derived from uridine diphosphate *N*-acetylmuramyl pentapeptide. It has been demonstrated by hydrazinolysis (Ito and Strominger, 1964) and synthetic studies (Lanzillotti *et al.*, 1964) that in this precursor the γ-carboxyl of D-glutamic acid is linked to the α-amino group of L-lysine. The α-carboxyl group of glutamic acid in this precursor is unsubstituted. The purpose of the present paper of which a preliminary account has appeared (Tipper and Strominger, 1965), is to demonstrate that the α-carboxyl group of glutamic acid is present as an amide in peptides obtained from cell walls of *S. aureus* and *A. crystallopoietes*, and carries a glycine residue with a free carboxyl group in a peptide obtained from cell walls of *M. lysodiekticus*.[1] These data also indicate that the glutamyl-lysine linkage in the cell wall peptides is a γ-glutamyl linkage, as it is in the nucleotide precursor, and that the peptidoglycans of these cell walls have in common the sequence N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysine. The

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‡ Supported in part by a grant from the U. S. Department of Agriculture under P.L. 480 (UR-E4-10-3).

[1] The isolation and characterization of two minor glycopeptide fractions from lysozyme digestion of *M. lysodeikticus* cell walls has been recently reported (Mirelman and Sharon, 1966). Linkage of glycine to the α-carboxyl of glutamic acid in these fractions was demonstrated by hydrazinolysis (added in proof).

presence of isoglutamine residues in the cell wall peptidoglycans of *Micrococcus roseus* and *Streptococcus pyogenes* has been more recently demonstrated by the isolation from all of them of the tetrapeptide N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine, which has been fully characterized (Muñoz *et al.*, 1966b).

Materials and Methods

Analytical Procedures. Procedures for the determination of free, NH_2-terminal, and total amino acids, total phosphate, and total hexosamine have been previously described (Ghuysen *et al.*, 1966b). Total amino acids were also determined by use of a Beckman-Spinco amino acid analyzer.

Bound (amide) ammonia in the original peptides was measured by the amino acid analyzer as the difference between the free ammonia content of a hydrolysate of a given peptide and that of an equal aliquot of peptide dissolved in the same volume of 4 N HCl, but frozen rather than heated during the period of hydrolysis. Bound ammonia in the products of Edman degradation was measured by the difference in free ammonia before and after hydrolysis for 6 hr in 4 N HCl at 100°.

Free ammonia was measured by a micromodification of the procedure of Ternberg and Hershey (1960). The diffusion apparatus consisted of a lyophilization vial (5-ml capacity) with a glass rod (3-mm diameter) extending through the stopper and about half way to the base of the vial. The sample (200 μl, 5–20 mμ-moles of ammonia) and saturated K_2CO_3 solution (200 μl) were placed in the vial, which was immediately stoppered after placing 5 μl of 2 N H_2SO_4 on the tip of the rod. The vials were held by clips to a slowly rotating board so that the rods were horizontal. After 1 hr at 10 rpm, the solutions on the rods were washed into tubes with water (5 × 20 μl). The washings were then partially neutralized by the addition of 2 N NaOH (4 μl), since excess acid affects the subsequent color production. Aliquots (20 μl) of these solutions were mixed with equal volumes (20 μl) of each of the color reagents (Ternberg and Hershey, 1960) to give a final volume of 100 μl. After standing for 1 hr at room temperature, water (300 μl) was added and the samples were read at 625 mμ. The recovery of ammonia after diffusion of samples of ammonium chloride was 98 ± 5%.

Column Fractionations. Columns (2 × 35 cm) of ECTEOLA- and CM-cellulose (Bio-Rad Cellex E and CM) were operated at room temperature at a flow rate of 0.5 ml/min. The columns were prewashed with 0.5 M LiCl and water before use. Unbound materials were eluted with several bed volumes of water, then bound materials were eluted with a linear gradient of LiCl concentration increasing up to 0.5 M. Pooled fractions containing LiCl were desalted by lyophilization and extraction with 100% ethanol, followed by gel filtration of the insoluble residues on Sephadex G-25, fine bead form (Pharmacia). Sephadex columns were operated at room temperature and eluted with water.

Peptides and free amino acids were quantitated in the eluates by measurement of free amino groups. Teichoic acids, polysaccharides, and disaccharides were quantitated by measurement of total phosphate and hexosamines.

Enzymes. The B enzyme from *Chalaropsis* was a gift from Dr. J. Hash of Lederle Laboratories (Hash, 1963; Tipper *et al.*, 1964). Acetylmuramyl-L-alanine amidase from *S. albus* G was prepared as previously described (Ghuysen *et al.*, 1962). The lytic peptidase from *Staphylococcus* K-6-W1 was a gift from the Mead Johnson Research Laboratories (Browder *et al.*, 1965). The lytic peptidase from *Myxobacter* strain AL-1 was a gift from Dr. J. C. Ensign (Ensign and Wolfe, 1965, 1966).

Cell Walls. S. aureus cell walls were prepared from early post-log-phase cells of strain Copenhagen as described previously (Tipper *et al.*, 1965). They were heated at 100° for 20 min to destroy autolytic enzymes before being digested with trypsin and ribonuclease. *A. crystallopoietes* cell walls were prepared from log-phase cells growing in the spherical form, as described previously (Ensign and Wolfe, 1965). *M. lysodeikticus* cell walls were prepared by differential centrifugation after disruption of the cells with glass beads at 4° in a Sorvall Omni-Mixer, and purified by trypsin digestion.

Peptides. A. THE POLYPEPTIDE OBTAINED BY HYDROLYSIS OF *S. aureus* CELL WALLS WITH THE ENDO-*N*-ACETYLMURAMIDASE FROM *Chalaropsis* AND THE ACETYLMURAMYL-L-ALANINE AMIDASE FROM *S. albus* G. This material (SA-Chal-amidase peptide) was prepared from isolated cell walls of *S. aureus* as described previously (Ghuysen and Strominger, 1963; Ghuysen *et al.*, 1965b). After lysis of the walls with the *Chalaropsis* B enzyme, the teichoic acid free soluble glycopeptide was isolated by paper electrophoresis. The eluted glycopeptide was purified by filtration on Sephadex G-50 and digested with the *S. albus* amidase. The resultant mixture of disaccharides and polypeptide was fractionated by filtration on Sephadex G-25 (see Ghuysen and Strominger, 1963, Figure 8).

B. PEPTIDE OBTAINED BY HYDROLYSIS OF *S. aureus* CELL WALLS WITH THE PEPTIDASE (LYSOSTAPHIN) FROM *Straphylococcus* K-6-W1. This material (SA-lysostaphin peptide) was prepared by digestion of isolated cell walls of *S. aureus* strain Copenhagen with a preparation of the peptidase from *Staphylococcus* K-6-W1 that was essentially free of hexosaminidase (Browder *et al.*, 1965). Cell walls (682 mg) and enzyme (1.9 mg) were incubated in 0.01 M potassium phosphate buffer (13.5 ml), pH 7.5, for 9 hr at 37°. The digest was desalted by filtration on Sephadex G-25 and fractionated on a column of ECTEOLA-cellulose. The basic peptide was eluted with water and then separated from uncharged materials by fractionation on a column of CM-cellulose. The peptide was eluted with LiCl as a single peak. The yield, after removal of LiCl, was 150 μmoles of peptide (as total glutamic acid) or about 50% of the theoretical yield (taking the molecular

weight of the repeating unit of the cell wall as 2080).

C. PEPTIDE OBTAINED BY HYDROLYSIS OF *S. aureus* CELL WALLS WITH THE PEPTIDASE (AL-1) FROM *Myxobacter* STRAIN AL-1. This material (SA-AL-1 peptide) was prepared as described previously (Tipper *et al.*, 1967). *S. aureus* strain Copenhagen cell walls (345 mg) were digested with *Myxobacter* AL-1 enzyme (0.46 mg) in 0.01 M sodium barbital buffer, pH 9.0 (7 ml), at 37° for 25 hr. The digest was then fractionated on a column of ECTEOLA-cellulose. Free alanine, neutral and basic peptides, and oligosaccharides were eluted by water in a single peak. The peptides were fractionated from the oligosaccharides by filtration on a column of Sephadex G-25 (2 × 60 cm), and were then fractionated on a column of CM-cellulose. The neutral peptides and free alanine were eluted with water and the basic peptides were subsequently eluted as a single peak with LiCl. The yield of basic peptides, after removal of LiCl, was 92 μmoles as total glutamic acid, or about 56% of the theoretical yield.

D. PEPTIDE OBTAINED BY HYDROLYSIS OF *A. crystallopoietes* CELL WALLS WITH THE PEPTIDASE (AL-1) FROM *Myxobacter* STRAIN AL-1. This material (AC-AL-1 peptide) was prepared as previously described (Tipper *et al.*, 1967). Cell walls of *A. crystallopoietes* (307 mg) were digested with *Myxobacter* AL-1 enzyme (1.2 mg) in 0.01 M sodium barbital buffer, pH 9.0 (6 ml), at 37° for 21 hr. The subsequent fractionation of the basic peptides from free amino acids, polysaccharides, and teichoic acid complex by chromatography on ECTEOLA- and CM-cellulose columns was essentially as described in the previous section. The yield of basic peptides was 64.5 μmoles, as total glutamic acid, or 52% of the theoretical yield, since these cell walls contain 0.41 μmole of glutamic acid/mg.

E. PEPTIDE OBTAINED BY HYDROLYSIS OF *M. lysodeikticus* CELL WALLS WITH THE PEPTIDASE (AL-1) FROM *Myxobacter* STRAIN AL-1. This material (ML-AL-1 peptide) was prepared by digestion of *M. lysodeikticus* cell walls (200 mg) with AL-1 enzyme (0.3 mg) from *Myxobacter* in 0.02 M sodium barbital buffer, pH 9.0 (10.5 ml), at 37° for 18 hr. The pH was then adjusted to 6 by the addition of 2 N HCl, and the mixture was heated for 5 min at 100° to destroy the enzyme. The pH was then adjusted to 7.5 by the addition of 0.1 M K_2HPO_4 (1.2 ml) and NaOH, and 0.3 mg of the *Staphylococcus* K-6-W1 endo-*N*-acetylglucosaminidase (containing some residual peptidase, *cf.* Browder *et al.*, 1965) was added. This procedure was employed first to split the peptide from the cell wall glycan with the AL-1 enzyme, and then to fragment the glycan into disaccharides with the K-6-W1 enzyme. After 8 hr, when the release of reducing power was maximal, the mixture was applied to a column of Sephadex G-25 and eluted with water. A single major peak, eluted just before salt, contained 85% of the reducing power and free amino groups of the original digest. This mixture was fractionated on a column of CM-cellulose. In contrast to the *S. aureus* and *A. crystallopoietes* peptides, all of the peptide in the *M. lysodeikticus* digest was eluted from this CM-cellulose column by water, in a peak that appeared just after the reducing power peak (which contained the disaccharide, *N*-acetylmuramyl-*N*-acetylglucosamine). The yield of peptide (30 μmoles) was 33% of the theoretical yield, since these cell walls contain 0.45 μmole of total glutamic acid/mg.[2]

F. SYNTHETIC PEPTIDES, L-isoglutamine (L-glutamic acid α-amide), α-glycyl-lysine, glycyl-glycyl-L-alanine, and L-alanyl-glycyl-glycine were purchased from Cyclo Chemical Corp. The purity of these materials was examined by paper electrophoresis at pH 6 and 2 followed by detection with ninhydrin. The concentrations of amino acids and ammonia in hydrolysates of standard solutions were measured using a Beckman-Spinco amino acid analyzer.

Edman Degradation Procedure. Konigsberg and Hill (1962) introduced the use of anhydrous trifluoracetic acid in the Edman degradation procedure, since it efficiently catalyzes the elimination of the 2-anilino-5-thiazolinone derivative of the NH_2-terminal amino acid from the phenylthiocarbamyl[3] derivative of a peptide, while causing no hydrolysis of peptide bonds. The sequence of a peptide with no repeated amino acids can thus be determined by qualitative analysis of the residual amino acids. A micromodification of this procedure was employed in the present studies, except that the results were analyzed by quantitative determination of the new NH_2-terminal amino acids.

1. COUPLING PROCEDURE. Lyophilized aliquots of peptides (0.1 μmole) were dissolved in *N*-ethylmorpholine buffer (Konigsberg and Hill, 1962) (60 μl) freshly prepared from redistilled *N*-ethylmorpholine. Phenylisothiocyanate (1 μl) was added. The mixtures were incubated at 37° for 45 min and water (87 μl) was then added. Excess reagent was extracted with ether (2 × 150 μl), and the solutions were lyophilized.

2. CYCLIZATION WITH PRODUCTION OF A NEW NH_2-TERMINAL AMINO ACID. The lyophilized PTC derivative was dissolved in trifluoroacetic acid (50 μl) and lyophilized after 45 min at room temperature. The residue was dissolved in 0.2 M acetic acid (90 μl) and extracted twice with benzene (150 μl) to remove the derivatives of the original NH_2-terminal amino acid. The residue was then analyzed for total NH_2-terminal amino acids with fluorodinitrobenzene.

3. PROCEDURE FOR TWO CYCLES OF EDMAN DEGRADATION OF CELL WALL PEPTIDES. Samples (0.05 μmole) of peptides derived from cell walls and of glycyl-glycyl-L-alanine and L-alanyl-glycyl-glycine were coupled with phenylisothiocyanate as described in section 1. The lyophilized PTC derivatives were cyclized in anhydrous trifluoroacetic acid as described in section 2. After 45 min at room temperature in sealed tubes, 4 N HCl (10 μl) was added to the trifluoroacetic acid solutions which were then lyophilized, redissolved in

[2] The preparation of this material was performed by Dr. Melina Leyh-Bouille (see Leyh-Bouille *et al.*, 1966).

[3] Abbreviations used: PTC, phenylthiocarbamyl derivative; FDNB, fluorodinitrobenzene; UDP, uridine diphosphate; ATP, adenosine triphosphate.

0.2 M acetic acid (90 μl), and extracted with benzene (2 × 150 μl). Aliquots (50 μl) were lyophilized for the second cycle of degradation. Aliquots were also analyzed for NH_2-terminal amino acids, free, and bound ammonia.

After one cycle of coupling and cyclization in trifluoroacetic acid, the residual peptides were subjected directly to a second coupling reaction without intermediate purification. The coupling procedure again was that described in section 1. Aliquots (0.3 μmole) of isoglutamine were also lyophilized for the second cycle of degradation. After cyclization in 4 N HCl (50 μl) for 4 hr at 30°, the solutions were extracted twice with benzene (100 μl) and aliquots were analyzed for NH_2-terminal amino acids, free ammonia, and bound ammonia. An isoglutamine sample which did not receive phenylisothiocyanate served as control for the nonspecific production of free ammonia (never more than 10%), while the treated isoglutamine sample served as a control for the efficiency of ammonia elimination.

Results

The Presence of Amide Ammonia in the Cell Wall Peptidoglycans. Total amino acid and ammonia analyses of the peptides employed indicated the presence of 1 mole of bound ammonia/mole of glutamic acid in each of the peptides derived from *S. aureus* and *A. crystallopoietes* (Table I). The *S. aureus* polypeptide (SA-Chal-amidase) and the peptide obtained on degradation of cell walls of *S. aureus* with lysostaphin each contained, in addition, 5 moles of glycine, 1 mole of lysine, and 2 moles of alanine, as does the intact peptidoglycan. The peptide obtained from *S. aureus* with the *Myxobacter* enzyme contained a reduced amount of glycine (1.7 moles). This material is a mixture of peptides containing one or two glycine residues.[4] Equivalent amounts of either tri- or tetraglycine are also released by this enzyme (Tipper *et al.*, 1967).

The peptide from *M. lysodeikticus*, on the other hand, contained no ammonia. A single glycine residue was present (Table I) in addition to one residue of lysine and two residues of alanine.

Earlier analyses of the composition of acid hydrolysates of the *S. aureus Chalaropsis* B glycopeptide had indicated that about 3 moles of ammonia were present/mole of glutamic acid. It was suggested that these were derived from the amino sugars which are extensively degraded under the conditions of hydrolysis employed (Ghuysen *et al.*, 1963). The presence of ammonia in the carbohydrate-free peptides cannot be explained on this basis, however. Moreover, the *Chalaropsis* B peptidoglycan migrated only slightly on electrophoresis at pH 3.8 and was not retarded by ECTEOLA-cellulose at neutral pH in the absence of salts (Ghuysen *et al.*, 1965b). The hexosamine-free polypeptide (SA-Chal-amidase peptide) prepared from it by treatment with the *S. albus* acetylmuramyl-L-alanine amidase, in common with the SA-lysostaphin, SA-AL-1, and AC-AL-1 peptides, could not be eluted from CM-cellulose by water and migrated toward the cathode at any pH between 2 and 10. Thus, all these peptides carry a net positive charge in this pH range. In contrast, the ML-AL-1 peptide was only slightly retarded by CM-cellulose in the absence of salts, and had no net charge at pH 5 as demonstrated by lack of electrophoretic migration. The presence of an amide in the cell wall peptidoglycans of the first two organisms, but not in the third, accounts for the observed differences in net charges on electrophoretic migration.

TABLE I: Total Amino Acid Analyses of Cell Wall Peptides.[a]

Peptide	Bound Ammonia	Lysine	Glutamic Acid	Glycine	Alanine
SA-Chal-amidase	107	107	100	530	210
SA-lysostaphin	81	105	100	512	198
SA-AL-1	108	100	100	170	210
AC-AL-1	130	117	100	5	215
ML-AL-1	0	94	100	110	199

[a] Data are expressed as moles/100 moles of glutamic acid. The abbreviations used to designate the peptides from *S. aureus* (SA), *A. crystallopoietes* (AC), and *M. lysodeikticus* (ML) are given in the text. Analyses were performed on a Beckman-Spinco amino acid analyzer with appropriate unhydrolyzed blanks for determination of bound ammonia (see text), with the exception of the SA-lysostaphin peptide which was analyzed by dinitrophenylation and quantitative thin layer chromatography. All of the materials were free of hexosamines with the exception of the SA-Chal-amidase peptide which contained about 10 moles each of glucosamine and acetylmuramic acid/100 moles of glutamic acid.

Edman Degradation of Isoglutamine. Isoglutamine (0.55 μmole) was coupled with phenylisothiocyanate as described (see Methods). Aliquots were removed at intervals and analyzed for residual isoglutamine by electrophoresis at pH 5.6 and detection with ninhydrin. Isoglutamine disappeared within 30 min at 37°, while there was no hydrolysis to glutamic acid in the absence of phenylisothiocyanate even after 2 hr at 37°. Thus the standard coupling conditions should give a quantitative yield of PTC-isoglutamine.

Since ammonia has a much lower pK than the amino group of a peptide, the acid-catalyzed elimination of ammonia from the PTC derivative of an α-

[4] D. Jarvis, unpublished experiments.

TABLE II: Edman Degradation of Synthetic Peptides.[a]

Peptide	Cycles of Edman Degradation	Ammonia		Amino-Terminal Amino Acids		
		Bound	Free	Glutamic Acid	Alanine	Glycine
Glycyl-glycyl-L-alanine	0				0	100
	1				0	98
	2				90	7
L-Alanyl-glycyl-glycine	0				100	0
	1				0	90
	2				0	87
Isoglutamine[b]	0	95		100		
	1	23	70	3		

[a] Data are expressed as moles/100 moles of initial NH_2-terminal amino acid. The degradation and analytical procedures are described in the text. The data are the averages of three separate experiments. [b] Employing the procedure for cyclization using 4 N HCl (see text) as in the second cycle of peptide degradation.

amino carboxamide is predictably much less efficient than the elimination of a shortened peptide from a PTC-peptide. Treatment of PTC isoglutamine (0.1 μmole) with trifluoroacetic acid (20 μl) at room temperature released only 0.02 μmole of ammonia after 6 hr, and only 0.03 μmole after 4 hr at 37°. However, treatment of PTC-isoglutamine with 4 N HCl at 30° resulted in the liberation of 65% of its ammonia at 4 hr, while under these conditions, only 10% of the ammonia was liberated from isoglutamine. This procedure was adopted for the cyclization step when testing for the presence of the PTC derivative of an α-aminocarboxamide, that is during the second cycle of degradation of cell wall peptides (see Methods).

The results presented for the cell wall peptides (see below) are the averages of at least three separate experiments, in all of which the recovery of free ammonia from an isoglutamine standard in the second cycle of degradation was 70 ± 5% (Table II). Most of the residual 30% remained as bound ammonia under these conditions. The efficiency of the two cycles for elimination of NH_2-terminal amino acids was determined from the yields of the expected new NH_2-terminal amino acids from the synthetic peptides, L-alanyl-glycyl-glycine and glycyl-glycyl-L-alanine (Table II). In every case, the yield of NH_2-glycine from Ala-Gly-Gly was 95 ± 5% at the end of the first cycle with no residual NH_2-alanine, and the yield of NH_2-alanine from Gly-Gly-Ala was 85 ± 10% at the end of the second cycle with never more than 5% residual NH_2-glycine.

Edman Degradation of the S. aureus Peptides. The SA-Chal-amidase polypeptide is a polymer with a low content of NH_2-terminal glycine (0.07 mole/mole of glutamic acid) corresponding to an average chain length of 15 peptide subunits. This peptide contains 0.82 mole of NH_2-terminal L-alanine and a small amount of NH_2-terminal glutamic acid, derived from peptide subunits that have lost their NH_2-terminal L-alanine residues. They may have been missing in the cell walls or else removed during subsequent enzyme treatments. The sum of NH_2-terminal alanine and NH_2-terminal glutamic is 0.9 residue. The polypeptide also contained about 0.1 disaccharide unit, not removed by the amidase. This fact presumably accounts for the 10% missing NH_2-terminal amino acids. The sum of the initial NH_2-glutamic acid of the peptide and that liberated after one cycle of degradation is also 0.9 mole. Neither of the other *S. aureus* peptides contains a significant amount of hexosamine. Hence, the sum of NH_2-terminal alanine and glutamic acid is 1.0 residue for both of them.

In the *S. aureus* polypeptide, in the first cycle of Edman degradation, the NH_2-terminal alanine disappeared and an equivalent amount of NH_2-terminal glutamic acid appeared (Table III). The initial NH_2-terminal glutamic acid also disappeared and an approximately equivalent amount of free ammonia appeared. The NH_2-terminal glycine remained constant during this cycle. During the second cycle of Edman degradation, most of the NH_2-terminal glutamic acid disappeared and free ammonia was liberated in an amount equal to 0.5 residue. The free ammonia liberated at the first cycle of reaction was volatilized during the second coupling procedure and thus the total ammonia liberated in the two cycles was equivalent to 0.62 residue. This value is in reasonable agreement with the yield of ammonia from isoglutamine itself (0.7 residue). Most of the remainder of the ammonia was present as bound ammonia at the end of the two cycles of reaction.

Similar data were obtained for the SA-lysostaphin and SA-AL-1 peptides. A small amount of ammonia was eliminated at the first cycle of reaction in both cases and a larger amount at the second cycle. In the SA-lysostaphin peptide the initial NH_2-terminal glycine

TABLE III: Edman Degradation of Cell Wall Peptides.[a]

Peptide	Cycles of Edman Degradation	Ammonia		NH_2-Terminal Amino Acids			
		Bound	Free	Glutamic Acid	Alanine	Glycine	ε-Lysine
SA-Chal-amidase	0	96	(36)[b]	8	82	7	0
	1	ND	12	83	0	8	4
	2	19	50	5	0	13	4
SA-lysostaphin	0	81	(5)[b]	10	90	46	3
	1	ND	5	80	2	47	5
	2	19	53	4	0	49	9
SA-AL-1	0	90	(4)[b]	12	87	75	5
	1	89	10	84	0	47	25
	2	13	48	3	0	10	28
AC-AL-1	0	106	(68)[b]	15	170	10	36
	1	90	20	88	0	0	78
	2	30	69	3	0	0	22
ML-AL-1	0	0	3	14	86	0	[c]
	1			80	0	4	
	2			0	0	83	

[a] Data are expressed as moles/100 moles of total glutamic acid in each peptide, and are the averages of at least three separate experiments. For details, see text. ND = not determined. [b] The free ammonia in the undegraded peptides is due to contaminating ammonium salts. It is volatilized in the first coupling reaction and so does not contribute to the subsequent analyses. [c] The measurement of free ε-amino groups of lysine in this particular analysis was inaccurate. Analyses presented in the following paper (Katz and Strominger, 1967) indicate that this peptide fraction contained 48 free ε-amino groups of lysine/100 glutamic acid residues. About 70% of these groups disappeared at the first cycle of reaction.

was 0.5 residue. It is believed that this compound is a monomer of the repeating subunit and should have an initial NH_2-terminal glycine of 1.0 residue. The low value is probably due to difficulty in determining NH_2-terminal glycine in long glycine peptides. In any case, the NH_2-terminal glycine did not change during two cycles of reaction with phenylisothiocyanate and thus the chain with an NH_2-terminal glycine must be at least three glycine residues long. The SA-AL-1 peptide had only 1.7 total glycine residues and gave an initial NH_2-terminal glycine of 0.75 residue. During the first cycle of degradation with phenylisothiocyanate, 0.28 residue of glycine disappeared and 0.25 residue of ε-amino-terminal lysine appeared. During the second cycle of reaction, 0.37 additional NH_2-terminal glycine disappeared and 0.28 additional ε-amino groups of lysine appeared. The ε-amino-terminal lysine which appears after the first cycle of degradation is reacted with phenylisothiocyanate in the second cycle and hence does not subsequently react with FDNB in the determination of free amino groups. Thus, the total of the initial ε-amino groups of lysine and those which appeared was 0.6, and 0.1 residue of NH_2-terminal glycine still remained, presumably substituted on lysine. Other data indicate that the SA-AL-1 peptide is a mixture of peptides containing different amounts of glycine substituted on the ε-amino group of lysine.[4]

In the degradation of the SA-Chal-amidase polypeptide and the SA-lysostaphin peptide, it should be noted that there was essentially no increase of NH_2-terminal lysine or of free amino groups of glycine during the second cycle of degradation. These data indicate firstly that there was very little random hydrolysis of peptide bonds during cyclization in 4 N HCl, and secondly that not more than 5% of the α-carboxyl groups of glutamic acid could have been substituted by glycine or by the α- or ε-amino groups of lysine. These data thus indicate that the glycine cross bridges cannot be linked to the α-carboxyl group of glutamic acid and that all of the glutamic acid is linked to lysine through the γ-carboxyl group. The latter fact had already been established for the glutamyl-lysine linkage in the uridine nucleotide precursor of the peptidoglycan. The proposed structures for the three peptides are shown in Figure 1.

Edman Degradation of the A. crystallopoietes Peptide. Analyses (Tipper *et al.*, 1967) have shown that this material has 1.8 moles of L-alanine/mole of glutamic acid, and 0.25 mole of D-alanine. After dinitrophenyla-

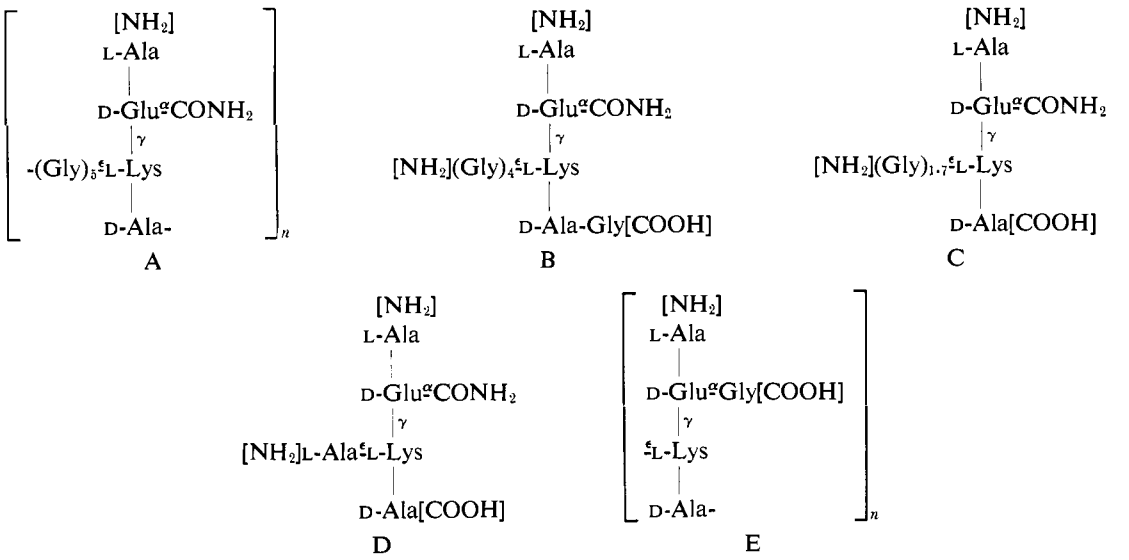

FIGURE 1: Proposed structures of the peptides. (A) SA-Chal-amidase polypeptide. In 10% of the repeating units, disaccharide is still substituted on L-alanine. In 8%, the L-alanine residue is missing so that glutamic acid is the NH_2 terminus. The chain length is about 15 units. (B) SA-lysostaphin peptide. The exact glycyl-glycine linkage split by lysostaphin is not known, and in fact this material could contain isomeric peptides resulting from hydrolysis of several different glycyl-glycine linkages. Again some of the peptides lack the NH_2-terminal L-alanine. (C) SA-AL-1 peptide. Much of the pentaglycine chains have been removed as tri- and tetraglycine. Again, 12% of these monomers have NH_2-terminal glutamic acid. (D) AC-Al-1 peptide. This is the monomer of the *Arthrobacter* cell wall peptide. Only a fraction of the material isolated has the COOH-terminal D-alanine residue and only part has the NH_2-terminal L-alanine substituted on the ϵ-amino group of lysine. (E) ML-AL-1 peptide. This material is a mixture of oligomers, where $n = 2$ and either 3 or 4 (Katz and Strominger, 1967).

tion and hydrolysis, 1.7 moles of DNP-alanine was produced and 0.3 mole of alanine remained (Table III). This material also contained 0.15 residue of NH_2-terminal glutamic acid and 0.36 residue of free ϵ-amino groups of lysine. The total NH_2-terminal amino groups were 2.2 residues/peptide subunit.

After one cycle of reaction with phenylisothiocyanate, no NH_2-terminal alanine remained, and approximately equal amounts of NH_2-terminal glutamic acid and ϵ-NH_2-lysine were liberated. Therefore, one of the L-alanine residues is substituted on the amino group of glutamic acid and the other on the ϵ-amino group of lysine. Together with experiments which showed that the *Myxobacter* enzyme hydrolyzes the cell wall of *A. crystallopoietes* with simultaneous production of NH_2-terminal L-alanine and COOH-terminal D-alanine (Tipper *et al.*, 1967), these data indicate that in the cell wall the extra L-alanine residue is present in an interpeptide bridge linking D-alanine and the ϵ-amino group of lysine. A small amount of free ammonia was also produced during the first cycle of reaction in an amount approximately equal to the initial NH_2-terminal glutamic acid.

During the second cycle of reaction, the NH_2-terminal glutamic acid produced after the first cycle disappeared with production of 0.69 residue of free ammonia. The high yield of ammonia in the two reaction cycles indicates that virtually all of the α-carboxyl groups of glutamic acid are amidated in this peptide also.

A probable structure for the major component of this peptide fraction is given in Figure 1D. About 15% of the peptides in the material have no L-alanyl substituent on glutamic acid and about 30% have no L-alanyl substituent on the ϵ-NH_2-group of lysine. The position of the 0.3 mole of alanine which is not NH_2 terminal has not been determined; it is presumably the COOH-terminal D-alanine present in some of the peptides.

Edman Degradation of the M. lysodeikticus Peptide. This material contained 0.86 residue of NH_2-terminal alanine and 0.14 residue of NH_2-terminal glutamic acid/residue of glutamic acid. It contained no bound ammonia, and so had no amide group. It did, however, contain 1 mole of COOH-terminal glycine/mole of glutamic acid (Katz and Strominger, 1967). This fact led to the prediction that, if the glycine were a substituent of the α-carboxyl group of glutamic acid, it would be liberated as free glycine after two cycles of Edman degradation.

After the first cycle of reaction, NH_2-terminal alanine disappeared and NH_2-terminal glutamic acid appeared in equivalent amounts. In addition, a small amount of NH_2-terminal glycine appeared.

After the second cycle of reaction, NH_2-terminal glutamic acid disappeared and an equivalent amount

of NH_2-terminal glycine appeared. This NH_2-terminal glycine was shown to be free glycine by the fact that, after reaction with FDNB, it was quantitatively extracted into ether without prior acid hydrolysis.

The proposed structure of the repeating unit of this peptide fraction is shown in Figure 1E. A small amount of the material is lacking its NH_2-terminal L-alanine residue. A fuller study of the components of this fraction is presented in the following paper (Katz and Strominger, 1967).

Discussion

The sequence of degradation with phenylisothiocyanate has, therefore, established that L-alanine is the first amino acid in the peptide sequence in each of the peptides, that D-glutamic acid is the second, and that the α-carboxyl group of glutamic acid is substituted as an amide in *S. aureus* and *A. crystallopoietes* and by a glycine residue with a free carboxyl group in *M. lysodeikticus*. Thus, in all of these cases the γ-carboxyl group of glutamic acid must be linked to the α-amino group of L-lysine. Thus, the data also indicate that the tetrapeptide subunit of the peptidoglycan is similar in structure in the three organisms examined. More recently, use of the peptidases produced by *S. albus* has led to the isolation of the tetrapeptide, N^{α}-(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine from cell walls of *S. aureus*, *M. roseus*, and *S. pyogenes*. The structure of these peptides was demonstrated by partial enzymic degradation, by specific chemical conversion of the isoglutamine residues to γ-aminobutyric acid, and by chromatographic comparison with synthetic peptides (Muñoz *et al.*, 1966b). Thus, these observations confirm and extend those reported here.

The data presented in this paper also indicate that no peptides are linked to the α-carboxyl group of D-glutamic acid in the cell wall peptidoglycans of these organisms, indirectly showing that their peptide subunits must be interlinked through their D-alanine carboxyl groups. This type of linkage was directly demonstrated in concurrent studies on the mode of action of the L_{II} enzyme from *Flavobacterium* on the cell walls of *S. aureus*. Lysis was accompanied by the release of NH_2-terminal glycine with parallel release of both COOH-terminal glycine and COOH-terminal D-alanine (0.73 and 0.37 mole/mole of total glutamic acid, respectively) (Kato *et al.*, 1962; K. Kato and J. L. Strominger, in preparation, 1967). A much higher yield of COOH-terminal D-alanine was obtained in studies on the mode of action of the lytic peptidases from *S. albus* G, demonstrating that most if not all of the interpeptide linkages in *S. aureus*, *M. lysodeikticus*, and *M. roseus* involve the carboxyl group of D-alanine (Petit *et al.*, 1966). Lysis of cell walls of *S. aureus* by the SA-endopeptidases was accompanied by the release of equal amounts of COOH-terminal D-alanine and NH_2-terminal glycine, and all the subsequently isolated peptides had COOH-terminal D-alanine. Lysis of cell walls of *M. lysodeikticus* by the ML-endopeptidase was accompanied by the simultaneous liberation of COOH-terminal alanine and free ϵ-amino groups of lysine. Lysis of cell walls of *M. roseus* was accompanied by the simultaneous liberation of COOH-terminal alanine and NH_2-terminal L-alanine (Petit *et al.*, 1966). Lysis of cell walls of *S. aureus* by the *Myxobacter* AL-1 enzyme was subsequently shown to be accompanied by the release of 1 mole of COOH-terminal D-alanine/mole of total glutamic acid (Tipper *et al.*, 1967). In *A. crystallopoietes*, the *Myxobacter* enzyme liberates COOH-terminal D-alanine and NH_2-terminal L-alanine simultaneously (Tipper *et al.*, 1967). The action of this enzyme, however, may be more complex on this substrate since it appears also to liberate some free D-alanine with liberation of COOH-terminal L-lysine (Tipper *et al.*, 1967, see figure 2B). This phenomenon is being further investigated and presumably accounts for the low content of D-alanine in the AC-AL-1 peptide. The action of bridge-splitting enzymes on other bacterial cell walls is also known to result in liberation of COOH-terminal D-alanine (see Strominger and Ghuysen, 1967). Thus, this appears to be the point of attachment of the peptide bridge in all organisms examined so far. The structural studies which are summarized here led to a detailed hypothesis of the mechanism by which penicillin interferes with the formation of these peptide bridges (Tipper and Strominger, 1965; Strominger and Tipper, 1965).

The occurrence of an amide of D-glutamic acid in the cell wall of *S. aureus* and *A. crystallopoietes* indicates that the second amino acid in the sequence is D-isoglutamine. This is the first report of the occurrence of either of the isomers of isoglutamine in a natural material. This compound is synthesized enzymatically during the reaction sequence in which the two uridine nucleotides, UDP-acetylmuramyl pentapeptide and UDP-acetylglucosamine, are utilized for bacterial cell wall synthesis. The substrates for amidation are disaccharide (-pentapeptide)-P-P-phospholipid, ATP, and ammonia.[5] With enzymes from *M. lysodeikticus*, glycine is added to the same lipid acceptor in a similar reaction (Katz *et al.*, 1967).

References

Browder, H. P., Zygmunt, W. A., Young, J. R., and Tavormina, P. A. (1965), *Biochem. Biophys. Res. Commun. 19*, 383.

Ensign, J. C., and Wolfe, R. S. (1965), *J. Bacteriol. 90*, 395.

Ensign, J. C., and Wolfe, R. S. (1966), *J. Bacteriol. 91*, 524.

Ghuysen, J.-M., Leyh-Bouille, M., and Dierickx, L. (1962), *Biochim. Biophys. Acta 63*, 286.

Ghuysen, J.-M., Petit, J. F., Munoz, E., and Kato, K. (1966a), *Federation Proc. 25*, 410.

Ghuysen, J.-M., and Strominger, J. L. (1963), *Biochemistry 2*, 1110.

[5] This work has been carried out by Dr. Gerhard Siewert (see Strominger *et al.*, 1966).

Ghuysen, J.-M., Tipper, D. J., Birge, C., and Strominger J. L. (1965a), *Biochemistry 4*, 2245.
Ghuysen, J.-M., Tipper, D. J., and Strominger, J. L. (1965b), *Biochemistry 4*, 474.
Ghuysen, J.-M., Tipper, D. J., and Strominger, J. L. (1966b), *Methods Enzymol. 8*, 685.
Hash, J. H. (1963), *Arch. Biochem. Biophys. 102*, 379.
Ito, E., and Strominger, J. L. (1964), *J. Biol. Chem. 239*, 210.
Kato, K., Kotani, S., Matsuhara, T., Kogami, J., Hashimoto, S., Chimori, M., and Kazekawa, I. (1962), *Biken's J. 8*, 155.
Katz, W., Matsuhashi, M., Dietrich, C. P., and Strominger, J. L. (1967), *J. Biol. Chem.* (in press).
Katz, W., and Strominger, J. L. (1967), *Biochemistry 6*, 930 (this issue; following paper).
Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.
Lanzillotti, A. E., Benz, E., and Goldman, L. (1964), *J. Am. Chem. Soc. 86*, 1880.
Leyh-Bouille, M., Ghuysen, J.-M., Tipper, D. J., and Strominger, J. L. (1966), *Biochemistry 5*, 3079.
Mandelstam, M. H., and Strominger, J. L. (1961), *Biochem. Biophys. Res. Commun. 5*, 466.
Mirelman, D., and Sharon, N. (1966), *Biochem. Biophys. Res. Commun. 24*, 237.
Muñoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J. F., and Tinelli, R. (1966a), *Biochemistry 5*, 3091.
Muñoz, E., Ghuysen, J.-M., Leyh-Bouille, M., Petit, J. F., Heymann, H., Bricas, E., and LeFrancier, P. (1966b), *Biochemistry 5*, 3748.
Petit, J. F., Muñoz, E., and Ghuysen, J.-M. (1966), *Biochemistry 5*, 2764.
Salton, M. R. J., and Ghuysen, J.-M. (1960), *Biochim. Biophys. Acta 45*, 355.
Strominger, J. L., and Ghuysen, J.-M. (1967), *Science* (in press).
Strominger, J. L., Matsuhashi, M., Anderson, J. S., Dietrich, C. P., Meadow, P. M., Katz, W., Siewert, G., and Gilbert, J. M. (1966), *Methods Enzymol. 8*, 473.
Strominger, J. L., and Tipper, D. J. (1965), *Am. J. Med. 39*, 708.
Ternberg, J., and Hershey, F. B. (1960), *J. Lab. Clin. Med. 56*, 707.
Tipper, D. J., Ghuysen, J.-M., and Strominger, J. L. (1965), *Biochemistry 4*, 468.
Tipper, D. J., and Strominger, J. L. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1133.
Tipper, D. J., Strominger, J. L., and Ensign, J. C. (1967), *Biochemistry 6*, 906 (this issue; preceding paper).
Tipper, D. J., Strominger, J. L., and Ghuysen, J.-M. (1964), *Science 146*, 781.