The Immunologically Active Cell Wall Peptide Polymer of *Staphylococcus aureus**

Kazuhito Hisatsune,† Samuel J. De Courcy, Jr., and Stuart Mudd

ABSTRACT: An immunologically active peptide component, P1, obtained from the centrifugal supernatants of cultures of the viscid Wiley wound strain of *Staphylococcus aureus* has been characterized as a cell wall peptide polymer. In gel filtration, the P1 was eluted in the imbibed volume through Sephadex G-50 (exclusion limit molecular weight, 10,000), and was eluted in the void volume through Bio-Gel P-2 (exclusion limit molecular weight, 1600). D-Glutamic acid, D- and L-alanine, L-lysine, and glycine were found as the component amino acids in the acid hydrolysate; in addition, considerable amounts of NH_3 also were found. The molar ratios were 1:1:2:5:1 for Glu-Lys-Ala-Gly-NH_3. Alanine was identified as the N-terminal residue, and no significant free NH_2 groups other than that of alanine were detected. The results obtained indicate the presence of the amide ($CONH_2$) structure of D-isoglutamine in this peptide, and also the presence of cross-linking between the ϵ-NH_2 groups of the lysine residues and terminal COOH groups, other than α-COOH groups of the glutamic acid residues, probably through pentaglycyl peptide bridges.

The P1 peptide was strongly immunologically active in precipitin reactions and showed a single, sharp precipitin band in immunoelectrophoresis with hyperimmune anti-Wiley serum and its homologous globulin. This serological activity was remarkably reduced by digestion with a Staphylococcal cell wall lytic enzyme, "lysostaphin."

The cell wall "mucopeptide" is believed to exist in the cell walls of all bacterial species as a "basal structure" (Salton, 1964). This mucopeptide consists of two primary structural components; one component is the *N*-acetylglycosamine-*N*-acetylmuramic acid polysaccharide backbone, and the other is the so-called "cell wall peptide" which is linked to the carboxylic group of muramic acid through a peptide linkage.

Although the antigenicity of the "cell wall mucopeptide" had been anticipated for a long time, it has only recently been reported in the case of streptococcal mucopeptide (Abdulla and Schwab, 1965; Karakawa and Krause, 1966). As to the cell wall peptide, on the other hand, an immunologically active cell wall peptide polymer, P1, has been obtained from centrifugal supernatants of cultures of the viscid "wound" strain of *Staphylococcus aureus* (Hisatsune *et al.*, 1967). The chemical and immunological characteristics of this cell wall peptide polymer are presented in this paper.

Materials and Methods

Preparation of the Cell Wall Peptide Polymer P1. The cell wall peptide polymer, P1, used in this study was prepared as described previously (Hisatsune *et al.*, 1967).

Gel Filtration. Sephadex G-50 (fine size, bead type) (Pharmacia Fine Chemicals, Rochester, Minn.) and Bio-Gel P-2 (100–200 mesh) (Bio-Rad Laboratories, Richmond, Calif.) were used for gel filtration at 2°.

Ultracentrifugation. Ultracentrifugation was carried out using the Spinco analytical ultracentrifuge, type E.

Chemical Analysis. The qualitative and quantitative amino acid analysis of the P1 peptide was accomplished by hydrolysis in constant boiling hydrochloric acid at 105° for 24 hr, followed by the technique of Spackman *et al.* (1958) using a Technicon AutoAnalyzer. *Reducing sugar* activity was measured according to the method of Momose *et al.* (1960a,b) using 3,6-dinitrophthalic acid. *Amino sugar* was determined by the method of Belcher *et al.* (1954), and the *organic and inorganic phosphorous* by the method of Allen (1940). The free NH_2 group reaction described by Ghuysen and Strominger (1963) was used, with slight modification, to detect the elution peak of the P1 component in gel filtration, and also for the estimation of each isolated amino acid (except lysine) by paper chromatography. To 0.05 ml of the sample, 0.2 ml of 1% $Na_2B_4O_7$ and 0.025 ml of 102 mM dinitrofluorobenzene (DNFB)[1] in ethanol were added. Each tube

* From the Department of Public Health and Preventive Medicine, School of Medicine, University of Pennsylvania, Department of Clinical Pathology of the Philadelphia General Hospital, and the U. S. Veterans Administration Hospital, Philadelphia, Pennsylvania. *Received November 7, 1966.* The preliminary report of this study appeared in *Biochim. Biophys. Acta 121*, 210 (1966). This investigation was supported by U. S. Public Health Service Grant A1 05473 and by the U. S. Veterans Administration Central Office Research Service.

† Fellow of the Theresa F. and Joseph Felsen Memorial Fund.

[1] Abbreviations used: DNFB, dinitrofluorobenzene; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; DAPA, diaminopimelic acid; "Anti-Wiley serum" means antistaphylococcal (Wiley) serum; "Anti-Wiley γ-globulin" means antistaphylococcal (Wiley) γ-globulin.

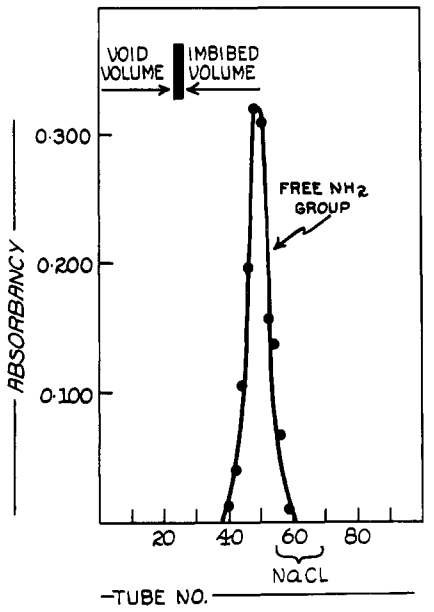

FIGURE 1: Elution pattern of Sephadex G-50 gel filtration of the P1. The P1 (102 mg in 3 ml of water) was placed on a Sephadex G-50 column (1.8 × 64 cm) and eluted with water. Fractions (3.2 ml) were collected automatically at a flow rate of 0.32 ml/min.

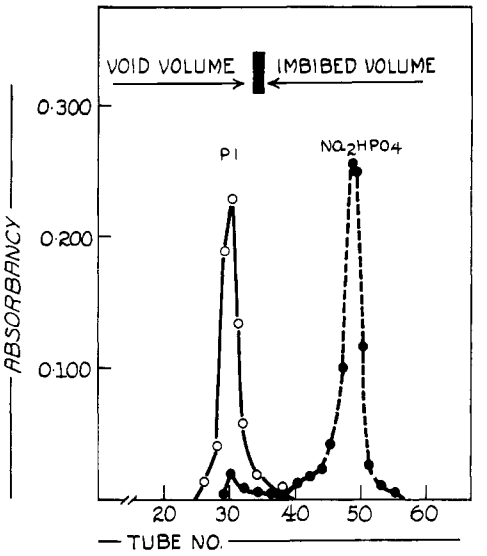

FIGURE 2: Elution pattern of Bio-Gel P-2 gel filtration of the P1. The P1 (18 mg plus 5 mg Na_2HPO_4 in 1.5 ml of water) was placed on a Bio-Gel P-2 (1.5 × 60 cm) and eluted with water. Fractions (1.8 ml) were collected automatically at a flow rate of 0.4 ml/min. (O—O—O), free NH_2 groups. (●- - -●- - -●), inorganic phosphate.

was immediately mixed on addition of DNFB, then heated at 60° for 30 min followed by the addition of 1.0 ml of 2 N HCl. Absorption was measured at 420 mμ. The *ninhydrin reaction* was used to measure lysine according to the method of Tsukamoto *et al.* (1957).

Determination of Optical Configuration. In the determination of the optical configuration of the component amino acid, L-glutamic acid was estimated using L-glutamic dehydrogenase (from bovine liver, crystalline, in sodium phosphate–glycerol solution, Sigma Chemical Co., St. Louis, Mo.) according to the method of Adelstein and Vallee (1958) and Strecker (1955). L-Lysine was estimated using L-lysine decarboxylase (from *B. cadaveris*, crude acetone powder, Sigma Chemical Co., St. Louis, Mo.) according to the method of Pelzer (1962). D-Alanine was estimated using D-amino acid oxidase (from pig kidney, benzoate complex, crystalline suspension in 0.8 M ammonium sulfate, Boehring Manheim Corp., New York, N. Y.) according to the method of Pelzer (1962) with modification. The preparation method of the apoenzyme of D-amino acid oxidase was based on the method described by Friedman (1963). L-Alanine was estimated using L-alanine dehydrogenase (Yoshida and Freese, 1964, 1965; Yoshida, 1965). Highly purified L-alanine dehydrogenase was supplied through the courtesy of Dr. Yoshida, Department of Genetics, School of Medicine, University of Washington, Seattle. In the determination of L-lysine and D-alanine, the reaction mixtures of isolated lysine or alanine with enzymes were spotted on paper after the enzyme reaction was completed, and then developed using pyridine–BuOH–AcOH–water (60:40:20:6, v/v) as the solvent system in descending paper chromatography. Amounts of amino acids on paper, which were not consumed by enzyme, were measured directly, without elution, using the ninhydrin reaction with an error of within 2–5% according to Tsukamoto and Komori (1960) and Tsukamoto *et al.* (1961).

N-Terminal Determination. The determination of the N-terminal amino acid residue was performed by dinitrophenylation using DNFB and triethylamine (Sanger and Thompson, 1953; Fraenkel-Conrat *et al.*, 1955). Resulting *N*-DNP-amino acid was identified by thin layer chromatography using Eastman chromatogram sheet type K 301R (silica gel) (Distillation Products Industries, Rochester, N. Y.).

Immunological Study. Hyperimmune anti-Wiley rabbit serum was obtained as described previously (Mudd and De Courcy, 1965). Homologous anti-Wiley γ-globulin was prepared from the corresponding high titer rabbit sera according to the method of Cherry *et al.* (1960). Antiserum and γ-globulin against *Micrococcus lysodeikticus* and *Bacillus subtilis* were prepared similarly. Lysostaphin (Schindler and Schuhardt, 1964) was supplied through the courtesy of Dr. P. A. Tavormina, Mead Johnson Research Center, Evansville, Ind. For digestion, 1 mg of the P1 was incubated with 200 μg of lysostaphin in 1 ml of 0.067 M phosphate-buffered saline (pH 7.2) for 6 hr at 37°. After incubation, the reaction mixture was tested in the precipitin reaction, both without heating the reaction mixture and after heating the mixture in boiling

water for 30 min to inactivate the enzyme and centrifuging to remove insoluble denatured enzyme. Precipitin reactions were performed in glass capillary tubes. Absorption tests on human agglutinin were carried out using the method followed by Lenhart *et al.* (1963). Immunoelectrophoresis was performed as described (Hisatsune *et al.*, 1967).

Results

Gel Filtration. In order to approximate the molecular size of P1, gel filtration through Sephadex G-50 and Bio-Gel P-2 was performed. The typical pattern obtained by Sephadex G-50 gel filtration of the component is shown in Figure 1. It was found that P1 through Sephadex G-50 was eluted in the imbibed volume, while in Bio-Gel P-2 gel filtration, P1 was eluted in the void volume (Figure 2). Ultracentrifugation of an aqueous solution of P1 at 52,640 rpm, 20°, failed to form a boundary. These results were interpreted to indicate that P1 is of a rather small molecular size, less than 10,000 but more than 1600 in molecular weight, approximately.

To determine components other than amino acids, the P1 component was hydrolyzed at 100° in 2 N HCl for 2 hr and 4 N HCl for 10 hr. It was found that the hydrolysates contained neither reducing sugar nor amino sugar components. No organic phosphorus was detected.

Without hydrolysis, P1 was found to be strongly positive for the free NH_2 group reaction using DNFB, and also for the ninhydrin reaction. Contamination by protein was checked by determining the ultraviolet absorption spectrum of a P1 aqueous solution (1 mg/ml). No characteristic absorption peak for protein was observed. This result further indicates that the isolated P1 peptide contains no aromatic amino acid.

Amino Acid Analysis. The results of amino acid analysis (Table I) show that only four component amino acids are present in the hydrolysate: glutamic acid, lysine, alanine, and glycine. In addition to these amino acids, a considerable amount of NH_3 was also found. Prolonged acid hydrolysis (48 hr) increased the molar ratio of glycine to 5.1:1 mole of glutamic acid. The molar ratios of all the amino acids and NH_3 were 1:1:2:5:1 for Glu-Lys-Ala-Gly-NH_3.

In addition to these four amino acids, small amounts of unknown ninhydrin-positive components were observed in the hydrolysate. One of these appeared to be serine as determined by comparative chromatography. The molar ratio of the "serine" component was 0.1:1 mole of glutamic acid. The other two unknowns were present in trace amounts. The results of amino acid analysis indicate that the P1 is a peptide. The molar ratios of the component amino acids of P1 (Table I) were found to coincide well with those of the mucopeptide fraction obtained from staphylococcal cell walls (Mandelstam and Strominger, 1961). It was suggested by Park and Strominger (1957) that the NH_3 which was present in the hydrolysate of the staphylococcal cell walls might have been derived from a degradation of the amino sugar component, muramic acid, during acid hydrolysis. However, since the P1 contains no amino sugar, the presence of considerable amounts of NH_3 in the hydrolysate indicates an amide structure in this peptide, most probably glutamine. Total recovery from the amino acid analysis was found to be more than 109% when calculated on the basis of total per cent content of each component amino acid (not residue) and NH_3.

TABLE I: Amino Acid Composition of the P1 and of the Mucopeptide Fraction of the Staphylococcal Cell Walls.

Amino Acid Residue Found	mμmoles	Molar Ratio	Molar Ratio of Mucopeptide Fraction[a]
Glutamic Acid	102.9	1	1
Lysine	103.9	1.00	0.85
Alanine	217.6	2.10	2.14
Glycine	468.3	4.55	4.61
(NH_3)	103.4	1.00	*b*
Total recovery (%)[c]	94.5		

[a] Quoted from the results by Mandelstam and Strominger (1961). [b] Not determined. [c] Calculated on the basis of total of amino acid residues per peptide hydrolyzed.

Optical Configuration of the Component Amino Acids. To determine whether D-amino acids were present in the hydrolysate of the P1 peptide, the optical configuration of each component amino acid was determined using highly stereospecific enzymes (Table II). The component amino acids in the hydrolysate were separated by paper chromatography and eluted, then each isolated amino acid was subjected to enzymatic assay.

Isolated glutamic acid was incubated with L-glutamic dehydrogenase in the presence of excess diphosphopyridine nucleotide (DPN) in the following system. A mixture of 0.5 ml of sample solution, 0.3 ml of 0.1 M Tris buffer, pH 8.0, and 0.2 ml of 0.04 M DPN solution was mixed with 0.005 ml of enzyme solution (5.7 mg/ml). Increase of absorbancy at 340 mμ for reduced diphosphopyridine (DPNH) was measured over a period of 150 min after addition of enzyme. As shown in Figure 3, the 100 mμM L-glutamic acid standard gave only 84% of theoretical reduction of DPN under these conditions. This may have been due to an unfavorable equilibrium in the reaction. The blank solution, D-glutamic acid standard solution, and as much as 7100 mμM isolated glutamic acid solution gave no increase in absorption. According to these results, the isolated glutamic acid was entirely D-glutamic acid.

In the L-alanine assay, the L-alanine standards were quantitatively consumed giving 102% of theoretical

TABLE II: Determination of Optical Configuration of the Component Amino Acids of the P1.

Isolated Amino Acid (mμmoles added)	Enzyme Used (mμmoles used by enzyme)	% Isomer	
		Found	Calcd[a]
Glutamic acid (7100)	L-Glutamic dehydrogenase (0)	0 L-Glu	100 D-Glu
Alanine (176)	L-Alanine dehydrogenase (90)	51 L-Ala	49 D-Ala
Alanine (118)	D-Amino acid oxidase (57–63)	48–53 D-Ala	47–52 L-Ala
Lysine (686)	L-Lysine decarboxylase (668)	>97 L-Lys	<3 D-Lys

[a] % isomer (calculated) = 100% − % isomer (found).

reduction of DPN, whereas isolated alanine gave 53% of theoretical reduction of DPN or 51% of that when corrected by control for L-alanine standard (Figure 3). In the L-lysine assay, more than 97% of isolated lysine was consumed by L-lysine decarboxylase, showing that isolated lysine was entirely L-lysine.

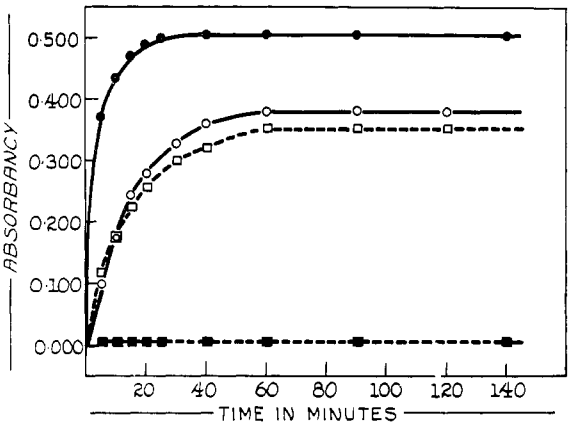

FIGURE 3: Time course of increase in absorbancy at 340 mμ in (A) L-glutamic acid assay with L-glutamic dehydrogenase and (B) L-alanine assay with L-alanine dehydrogenase. In L-glutamic acid assay, 0.2 ml of 0.04 M DPN solution, 0.3 ml of 0.1 M Tris buffer, pH 8.0, and 0.5 ml of sample solution (containing 7100 mμmoles of isolated glutamic acid or 100 mμmoles of L-glutamic acid standard) were mixed in a small-scale cuvet (light path length, 1 cm) and the absorbancy at 340 mμ was measured; 0.005 ml of L-glutamic dehydrogenase solution (5.7 mg/ml) was then added to the cuvet and the absorbancy was measured at different intervals. In L-alanine assay, 0.6 ml of 0.02 M DPN solution (neutralized), 0.6 ml of 0.1 M Na_2CO_3–$NaHCO_3$ buffer, pH 10.0, and 0.3 ml of sample containing 176 mμmoles of isolated L-alanine or 82 mμmoles of L-alanine standard were mixed in a cuvet and absorbancy at 340 mμ was measured; 0.005 ml of L-alanine dehydrogenase solution (2 mg/ml) was added to the cuvet and absorbancy was measured at different intervals. (●—●—●), L-glutamic acid standard. (■—■—■), isolated glutamic acid. (○—○—○), L-alanine standard. (□- - -□- - -□), isolated alanine.

Determination of the N-Terminal Amino Acid Residue. Determination of the N-terminal amino acid residue of the P1 was performed using the dinitrophenylation method. A considerable amount of the "ether-extractable *N*-DNP-amino acid fraction" obtained from 3 mg of the P1 was spotted on a silica gel plate in thin layer chromatography for the identification of the *N*-DNP-amino acid. Only one spot was observed in the three solvent systems which was identified as *N*-DNP-alanine (Table III). On the other hand, no significant spot was observed in the chromatogram of an "acid- and water-soluble *N*-DNP-amino acid fraction," with a solvent system (1-propanol–34% ammonia, 70:30 v/v) which is usually used for identification of ε-*N*-mono-DNP-lysine. Although the spotting of larger samples on the chromatoplate might show spots other than that for *N*-DNP-alanine, after chromatography, it was concluded from these results that

TABLE III: Thin Layer Chromatography of *N*-DNP-Amino Acid Residue of the P1.[a]

	Solvent System[b]		
	A	B	C[c]
	Solvent Migration (cm)		
	15	15	10
Sample	0.51	0.69	0.81
N-DNP-Ala (standard)	0.51	0.69	0.81
N-DNP-Glu (standard)	0.02	0.10	0.67
N-DNP-Gly (standard)	0.41	0.35	0.63
N-Di-DNP-Lys (standard)	0.82	0.74	0.75

[a] R_F value in ascending chromatography. [b] Solvent: (A) toluene–pyridine–ethyl chlorohydrin–0.8 N NH_4OH (100:30:66:60, v/v); (B) chloroform–MeOH–AcOH (95:5:1, v/v); and (C) chloroform–*t*-amylOH–AcOH (70:30:3, v/v). [c] R_F values based on DNP-leucine (standard) (R_F 1).

the N-terminal residue of the P1 is primarily alanine, and even if terminal residues other than alanine are present, they should be present in relatively very small amounts.

Serological Analysis of the P1. Strong immunological activity of the P1 was demonstrated by the precipitin reaction with hyperimmune anti-Wiley rabbit serum (Table IV). To test the possibility that serum components other than antibody might be reacting to produce the precipitates with the P1, γ-globulin was prepared from the homologous anti-Wiley serum and tested against the P1 in the precipitin reaction. The γ-globulin was redissolved in saline solution to a concentration corresponding to its original concentration in the serum. The result was the same as with the anti-Wiley serum. Sera and their γ-globulins obtained from rabbits which were hyperimmunized with *M. lysodeikticus* and *B. subtilis* failed to show any precipitin reaction with the P1.

TABLE IV: Precipitin Reaction of the P1 with Hyperimmune Anti-Wiley Serum and the Homologous Anti-Wiley γ-Globulin.

Antigen	Serum or γ-Globulin (undiluted)	Precipitin Titer of Antigen (reciprocal value × 10^{-3}) 24 hr	48 hr
P1	anti-Wiley serum	1024	4096
P1	anti-Wiley γ-globulin	1024	4096
P1	anti-*M. lysodeikticus* serum	<1	<1
P1	anti-*M. lysodeikticus* γ-globulin	<1	<1
P1	anti-*B. subtilis* serum	<1	<1
P1	anti-*B. subtilis* γ-globulin	<1	<1
P1 (digested with lysostaphin	anti-Wiley serum	<1	2
P1 (plus inactivated lysostaphin)	anti-Wiley serum	1024	4096

Digestion of 1 mg of the P1 with 200 μg of staphylococcal cell wall lytic enzyme, lysostaphin, remarkably reduced the serological activity (Table IV). When 0.4 ml of the anti-Wiley serum was absorbed with 12.5 μg of the P1 which was digested with lysostaphin, slight inhibition of the precipitin reaction was observed.

Since immunological results of lysostaphin digestion and the chemical nature of the P1 in this study indicated a close relationship to the cell wall of *Staphylococcus*,

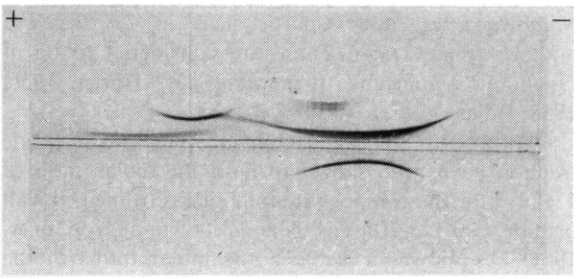

FIGURE 4: Immunoelectrophoretic pattern of the P1 with rabbit anti-Wiley γ-globulin. The aqueous P1 solution (1 mg/ml) was placed in the lower well, and the partially purified starting material solution (1 mg/ml in water) of the carbohydrate–peptide fraction of the *S. aureus* culture filtrates from which the P1 was isolated was placed in the upper well. Anti-Wiley γ-globulin reconstituted with saline solution to the original concentration in serum (82 mg/ml) was placed in the center trough. Phosphate buffer (0.1 M, pH 7.2), 200 v, 35 ma for 1 hr. The P1 band appears below the center trough (*cf.* Hisatsune *et al.* (1967); Figure 11c).

the effect of the P1 on agglutination of *S. aureus* with human agglutinins was studied. Human serum from a healthy donor was absorbed with the P1 (20 mg/ml serum, diluted 1:10); the absorbed and unabsorbed serum samples, tested by Miss N. A. Lenhart, showed no difference in agglutination of *S. aureus*. However, the antibody against the common protein agglutinogen (Lenhart *et al.*, 1963) would not have been removed by this absorption procedure, so that the presence of the P1 in trace amounts in the bacterial surface is not excluded. The sensitizing effect on sheep red cells (not tanned) tested against anti-Wiley rabbit serum also was found to be negative.

Immunoelectrophoresis. Immunoelectrophoresis was conducted with the P1, and a precipitin band in agar was developed against γ-globulin derived from potent anti-Wiley rabbit serum. Immunoelectrophoretic patterns identical with those obtained with anti-Wiley rabbit serum (Hisatsune *et al.*, 1967) were obtained with homologous γ-globulin, *i.e.*, a sharp, single, precipitin band which migrated toward the cathode (Figure 4).

Discussion

The proof that the P1 component which has been isolated in our study is the cell wall peptide polymer is based on the following established criteria. (1) P1 consists of four component amino acids: alanine, glutamic acid, lysine, and glycine. It has been generally accepted that the cell wall peptide usually consists of three component amino acids: alanine, glutamic acid, and lysine. Lysine is occasionally replaced by diaminopimelic acid (DAPA), depending on the bacterial species. In addition to these amino acids, other amino acids are also present in the mucopeptide of certain bacteria, for example, large amounts of glycine in

Staphylococcus, and aspartic acid in *Lactobacillus* and *M. lysodeikticus*. These are considered to be of taxonomic significance (Cummings and Harris, 1956, 1958; Rogers, 1962).

Hydrolysates of the staphylococcal mucopeptide fraction have been shown to have the molar ratio of 1:1:2:5 for the component amino acids of the cell wall peptide, Glu-Lys-Ala-Gly (Mandelstam and Strominger, 1961). As shown in Table I, the molar ratios of the component amino acids of P1 coincide well with these. (2) The glutamic acid residue of P1 is composed entirely of D-glutamic acid, and the alanine residue is composed of D- and L-alanine. The cell wall peptide is known to have a unique character in the optical configuration of the component amino acids, and it has been known that the glutamic acid residue is entirely D-glutamic acid and the alanine residue is D- and L-alanine, whereas the lysine residue is entirely L-lysine. The presence of the D isomers of glutamic acid and alanine and sometimes aspartic acid is considered to be a characteristic which distinguishes the cell wall peptide from other normally occurring substances, because natural peptides and proteins are usually composed of L isomers of amino acids with the exception of a few, for example, a special cyclic peptide, bacitracin A (Abraham and Newton, 1958). (3) The N-terminal residue is alanine. Studies on accumulation of nucleotide precursors of cell wall biosynthesis in *Staphylococcus* inhibited by penicillin have disclosed each step of the biosynthetic process of the mucopeptide, especially its peptide moiety (Park and Johnson, 1949; Park, 1952; Park and Strominger, 1957; Strominger and Threnn, 1959), leading to the general speculation that the amino acid sequence of the cell wall peptide of *Staphylococcus* is L-Ala-D-Glu-L-Lys-D-Ala. The accumulation of uridine diphosphate–*N*-acetylmuramyl-L-Ala (Park's nucleotide II) (Park, 1952) and the enzymic reaction of uridine diphosphate–*N*-acetylmuramic acid with L-alanine to form this nucleotide (Ito and Strominger, 1960) especially suggest that the cell wall peptide chain in the mucopeptide is linked to muramic acid through the peptide linkage between NH_2 groups of the N-terminal alanine residue of the peptide and the COOH group of muramic acid. Ghuysen (1957, 1960) has shown that the *Streptomyces* enzyme, F_2B, possesses an amidase activity which cleaves the linkage between muramic acid and the cell wall peptide moiety, liberating a free NH_2 group susceptible to dinitrophenylation. Using this enzyme, he showed that alanine is at the N-terminal end of the cell wall peptide of the *M. lysodeikticus* cell wall. Liberation of N-terminal alanine by the treatment of cell walls with the F_2B enzyme was also confirmed in several other organisms including *Staphylococcus* (Salton, 1961), leading to the general concept that alanine is the N-terminal residue of the cell wall peptide moiety which is joined to the COOH group of muramic acid to form the mucopeptide. On the other hand, a fragment consisting of muramic acid and L-alanine was found by the partial hydrolysis of "C_5 and C_6 unit," subunits of the pure mucopeptide of *E. coli* B cell walls, known to consist of *N*-acetylglucosamine, *N*-acetylmuramic acid, D-, and L-alanine, D-glutamic acid, and DAPA (Primosigh *et al.*, 1961; Pelzer, 1962). (4) No ϵ-NH_2 groups of the lysine residue of the P1 are dinitrophenylated in the reaction with DNFB. A further characteristic of the cell wall peptide is the unique cross-linkage structures which have been revealed by the unusual behavior of cell walls or mucopeptide to DNFB reagent in the dinitrophenylation reaction for detection of the free NH_2 group. It has been shown that not all ϵ-NH_2 groups of lysine (or DAPA) of the cell walls or their mucopeptide fractions react with DNFB (Ingram and Salton, 1957; Salton, 1961; Primosigh *et al.*, 1961; Weidel and Pelzer, 1964). This fact led to the suggestion that ϵ-NH_2 groups of lysine (or DAPA) are masked by being bound with amino acids or peptide, thus indicating the presence of cross-linkage in the cell wall peptide at the ϵ-NH_2 group of lysine (or DAPA). In *Staphylococcus*, the cell wall mucopeptide fraction after reaction with DNFB has been found to contain no α- or ϵ-NH_2 group of lysine and no free NH_2 group of the other amino acids except for trace amounts of free NH_2 group of glycine (Mandelstam and Strominger, 1961). Accordingly, Mandelstam and Strominger speculated that the cell wall peptide chain (consisting of D-Glu, L-Lys, and D- and L-Ala) of staphylococcal mucopeptide is cross-linked through the ϵ-NH_2 group of lysine in the one site and the γ-COOH group of D-glutamic acid of the adjacent chain in the other site, most likely by pentaglycyl peptide bridges. The behavior of the ϵ-NH_2 group of lysine of the P1 to DNFB is completely identical with that of the staphylococcal mucopeptide.

Based on the data from (1) amino acid composition, (2) optical configuration of the component amino acids, (3) N-terminal amino acid determination, and (4) reaction with DNFB, the results of this study are compatible with the interpretation that this P1 is a cell wall peptide polymer which is most probably cross-linked by pentaglycyl peptide bridges. Since the molecular weight for Glu_1-Lys_1-Ala_2-Gly_5 is calculated to be 703, the result of Bio-Gel filtration (exclusion limit molecular weight 1600) indicates that the P1 is not composed of a single minimal unit (L-Ala-D-Glu-L-Lys-D-Ala) of the cell-wall peptide but is rather a polymer of the repeating unit linked through pentaglycyl peptide bridges.

The purity of the isolated peptide is indicated by the total recovery from amino acid analysis, a single peak in chromatography, and a single sharp precipitin band in immunoelectrophoresis. The elution volume of the peptide in gel filtration and ultracentrifugation suggests that it has a rather small molecular size, a molecular weight less than 10,000 but more than 1600, approximately.

The amino acid analysis shows that 1 mole of NH_3:1 mole of glutamic acid is present in the hydrolysate of this peptide. The presence of NH_3 in the hydrolysate strongly indicates an amide structure ($CONH_2$) in the peptide so that the D-glutamic acid residue is present as D-isoglutamine, because, as mentioned before,

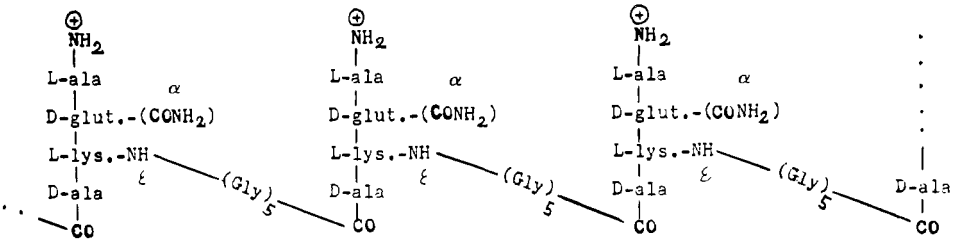

FIGURE 5: Proposed two-dimensional structure of the peptide polymer.

no amino sugar, in particular muramic acid, is contained in the P1, and so NH_3 is unlikely to occur from other than amide nitrogen of the peptide in the analytical techniques employed. This conclusion coincides well with that of Tipper and Strominger (1965) based upon the terminal disaccharide peptide unit of staphylococcal cell wall mucopeptide.

The presence of no other significant N-terminal amino acid residue than alanine suggests a high homogeneity in the internal structure of the peptide molecule. The fact that there is no significant amount of free ϵ-NH_2 groups of lysine detectable by dinitrophenylation with DNFB indicates the presence of cross-linkage, most likely through pentaglycyl peptide bridges between the ϵ-NH_2 group of lysine of the cell wall chain in the one site and some available C-terminal residue of the adjacent peptide chain at the other site. Substitution of α-COOH groups in D-glutamic acid with the amide structure ($CONH_2$) seems to exclude the possibility that this α-COOH group participates in the cross-linkage with the ϵ-NH_2 group of lysine of the adjacent peptide chain.

The following is considered to be a reasonable explanation of the considerable positive net charge possessed by the P1 peptide. The α-COOH group of the D-glutamic acid in the proposed structure of the cell wall peptide (NH_2-L-Ala-D-Glu-L-Lys-D-Ala-COOH) is amidated to form D-isoglutamine. The ϵ-NH_2 group of lysine in the peptide chain is cross-linked to the COOH terminal group of the D-alanine residue of the adjacent peptide chain through a pentaglycyl peptide bridge, leaving the NH_2-terminal group of the L-alanine residue free to give a positive net charge to the peptide (Figure 5). In this case, the proposal of Ghuysen, Tipper, Birge, and Strominger dealing with the pentaglycyl peptide cross-linkage in the staphylococcal mucopeptide seems to be most acceptable (Ghuysen *et al.*, 1965).

The P1 has been found to be immunologically active as shown in precipitin reactions with hyperimmune anti-Wiley serum. A positive precipitin reaction with γ-globulins derived from anti-Wiley serum seems to exclude the possibility of nonspecific reactions with serum components other than antibody.

A remarkable reduction of serological activity of P1 was observed after digestion with lysostaphin. Lysostaphin is a staphylococcal cell wall lytic enzyme (Schindler and Schuhardt, 1964; Schuhardt and Klesius, 1966), and is known to split the pentaglycyl peptide bridges (Browder *et al.*, 1965; Tipper and Strominger, 1966). This immunological result as to lysostaphin digestion is considered to be further evidence indicating that the P1 is the cell wall peptide polymer.

If the cell wall peptide were assumed to be exposed *in situ* on the cell surface, it should be anticipated that interaction of the peptide with its corresponding antibody would affect agglutination. However absorption of human serum with the peptide polymer did not detectably alter the agglutination titer of the serum (*cf.* Morse (1965), p 198). This result suggests that the peptide is not exposed at the cell surface, at least in significant amounts, a conclusion which is of course compatible with morphological studies on the degradation of the cell wall. Although the antigenicity of the cell wall mucopeptide has been anticipated for a long time, it has only recently been reported in the case of streptococcal mucopeptide (Abdulla and Schwab, 1965; Karakawa and Krause, 1966).

As far as the cell wall peptide is concerned, very little has been reported on the immunological characterization and much less on the chemical characterization of the materials used in such immunological studies, in an effort to prove that the materials were really the cell wall peptide. Since the preliminary report of this paper (Hisatsune *et al.*, 1966), an inhibitor of the precipitin reaction of streptococcal mucopeptide with its antiserum was obtained by the digestion of streptococcal mucopeptide with *Streptomyces albus* enzyme (Karakawa and Krause, 1966). This fraction was reported to consist of alanine, lysine, and glutamic acid in a molar ratio of 3:1:1.

A tanned cell-sensitizing component was reported by Oeding and his collaborators in their extensive immunological studies on *Staphylococcus* (Grov *et al.*, 1964; Oeding *et al.*, 1964; Oeding, 1965). This substance isolated from Jensen's antigen A (or extract A) of *S. aureus* (Jensen, 1958) was reported to consist of aspartic acid, alanine, lysine, glutamic acid, glycine, and serine, and to have no activity as precipitinogen. However, no detailed report of relationship with the cell wall peptide has been published except for brief qualitative citations of its component amino acids.

In the present study, the P1 is characterized as being the cell wall peptide polymer. Immunological activity of this peptide has been clearly demonstrated. It is

immunochemically interesting that a peptide of such small molecular size should show the precipitin reaction. This is thought to be the first report of isolation and purification of the cell wall peptide polymer and the first report of the immunological activity of the isolated cell wall peptide polymer. Further chemical characterization and immunological studies on the peptide polymer, especially the determination of the amino acid sequence, are under investigation.

Acknowledgment

The authors wish to express their appreciation to Dr. S. Utsumi for his aid in the amino acid analysis, Dr. A. Yoshida for his aid in the amino acid analysis and L-alanine determination and his courtesy in supplying us with L-alanine dehydrogenase, Dr. P. A. Tavormina for his courtesy in supplying us with lysostaphin, Miss Nancy A. Lenhart for aid in the inhibition test of agglutination, and Miss Sarvertia Askins and Miss Etta Jones for technical assistance.

References

Abdulla, E. M., and Schwab, J. H. (1965), *Proc. Soc. Exptl. Biol. Med. 118*, 359.

Abraham, E. P., and Newton, G. F. (1958), *Ciba Found. Symp., Amino Acids Peptides Antibiotic Activity*, 205.

Adelstein, S. J. and Vallee, B. L. (1958), *J. Biol. Chem. 233*, 589.

Allen, R. J. L. (1940), *Biochem. J. 34*, 858.

Belcher, R., Nutten, A. J., and Sambrook, C. M. (1954), *Analyst 79*, 201.

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

Cherry, W. B., Goldman, M., Carsky, T. R., and Moody, M. D. (1960), Fluorescent Antibody Techniques in the Diagnosis of Communicable Disease, Atlanta, Ga., Communicable Disease Center.

Cummings, C. S., and Harris, H. (1956), *J. Gen. Microbiol. 14*, 583.

Cummings, C. S., and Harris, H. (1958), *J. Gen. Microbiol. 18*, 173.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Analy. 2*, 359.

Friedman, C. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, N. U., Ed., New York, N. Y., Academic, p 598.

Ghuysen, J.-M. (1957), *Arch. Intern. Physiol. 65*, 173.

Ghuysen, J.-M. (1960), *Biochim. Biophys. Acta 40*, 473.

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

Ghuysen, J.-M., Tipper, D. J., Birge, C. H., and Strominger, J. L. (1965), *Biochemistry 4*, 2245.

Grov, A., Myklestad, B., and Oeding, P. (1964), *Acta Pathol. Microbiol. Scand. 61*, 588.

Hisatsune, K., De Courcy, S. J., Jr., and Mudd, S. (1966), *Biochim. Biophys. Acta 121*, 210.

Hisatsune, K., De Courcy, S. J., Jr., and Mudd, S. (1967), *Biochemistry 6*, 586 (this issue; preceding paper).

Ingram, V. M., and Salton, M. R. J. (1957), *Biochim. Biophys. Acta 24*, 9.

Ito, E., and Strominger, J. L. (1960), *J. Biol. Chem. 235*, PC5.

Jensen, K. (1958), *Acta Pathol. Microbiol. Scand. 44*, 421.

Karakawa, W. W., and Krause, R. M. (1966), *Bacteriol. Proc.*, 60.

Lenhart, N. A., Mudd, S., Yoshida, A., and Li, I. W. (1963), *J. Immunol. 91*, 771.

Mandelstam, M. H., and Strominger, J. L. (1961), *Biochem. Biophy. Res. Commun. 5*, 466.

Momose, T., Inaba, A., Mukai, Y., and Watanabe, M. (1960a), *Talanta 4*, 33.

Momose, T., Mukai, Y. and Watanabe, M. (1960b), *Talanta 5*, 275.

Morse, S. I. (1965), *Ann. N. Y. Acad. Sci. 128*, 191.

Mudd, S., and De Courcy, S. J., Jr. (1965), *J. Bacteriol. 89*, 874.

Oeding, P. (1965), *Ann. N. Y. Acad. Sci. 128*, 183.

Oeding, P., Grove, A., and Myklestad, B. (1964), *Acta Pathol. Microbiol. Scand. 62*, 117.

Park, J. T. (1952), *J. Biol. Chem. 194*, 877, 885, 897.

Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 179*, 585.

Park, J. T., and Strominger, J. L. (1957), *Science 125*, 99.

Pelzer, H. (1962), *Biochim. Biophys. Acta 63*, 229.

Primosigh, J., Pelzer, H., Maas, D., and Weidel, W. (1961), *Biochim. Biophys. Acta 46*, 68.

Rogers, H. J. (1962), *Biochem. Soc. Symp. 22*, 55.

Salton, M. R. J. (1961), *Biochim. Biophys. Acta 52*, 329.

Salton, M. R. J. (1964), The Bacterial Cell Wall, Amsterdam, Elsevier, p 133.

Sanger, F., and Thompson, E. O. P. (1953), *Biochem. J. 53*, 353.

Schindler, C. A., and Schuhardt, V. T. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 414.

Schuhardt, V. T., and Klesius, P. H. (1966), *Bacteriol. Proc., 70.*

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Strecker, H. J. (1955), *Method Enzymol. 2*, 220.

Strominger, J. L., and Threnn, R. H. (1959), *Biochim. Biophys. Acta 36*, 83.

Tipper, D. J., and Strominger, J. L. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1133.

Tipper, D. J., and Strominger, J. L. (1966), *Biochem. Biophys. Res. Commun. 22*, 48.

Tsukamoto, T. and Komori, T. (1960), *Chem. Pharm. Bull. (Tokyo) 8*, 913.

Tsukamoto, T., Komori, T., and Inoue, Y. (1961), *Yakugaku Zasshi 81*, 146.

Tsukamoto, T., Komori, T., and Kinoshita, N. (1957), *Chem. Pharm. Bull. (Tokyo) 5*, 363.

Weidel, W., and Pelzer, H. (1964), *Advan. Enzymol. 26*, 193.

Yoshida, A. (1965), *Anal. Biochem. 11*, 383.

Yoshida, A., and Freese, E. (1964), *Biochim. Biophys. Acta 92*, 33.

Yoshida, A., and Freese, E. (1965), *Biochim. Biophys. Acta 96*, 248.

Malate Dehydrogenases. I. A Survey of Molecular Size Measured by Gel Filtration*

William H. Murphey,† G. Barrie Kitto,‡ Johannes Everse, and Nathan O. Kaplan

ABSTRACT: The molecular sizes of some diphosphopyridine nucleotide linked malate dehydrogenases (including representatives of several major groups of animal, plant, and microbial species) have been determined by gel filtration. The elution volumes of the malate dehydrogenase activity in crude, cell-free extracts were identical with those of the partly purified enzymes and of the purified, crystalline enzymes. The elution volumes of malate dehydrogenases (mitochondrial and/or supernatant) from all animal and plant sources examined were equal, as were those of the enzymes from several microbial species; however, significantly smaller elution volumes, corresponding to higher molecular weights, were obtained for malate dehydrogenases of certain Gram-positive bacteria in the order *Eubacteriales*. Crystalline proteins, typical of small and large forms of malate dehydrogenase, were dissociated into enzymatically inactive subunits by treatment with acid, urea, or guanidine·HCl; partial reactivation was obtained by dialysis or dilution of the dissociating agent.

While the molecular weights of the diphosphopyridine nucleotide linked dehydrogenases range from 20,000 (dihydrofolate reductase) to more than 300,000 (beef liver glutamate dehydrogenase), little variation is usually found in specific dehydrogenases from one animal or tissue source to another, *e.g.*, the triosephosphate (Allison and Kaplan, 1964) and lactate dehydrogenases (Wilson *et al.*, 1964). On the other hand, the values published for the molecular weights of malate dehydrogenases from different sources would seem to indicate much greater variation in the molecular size of this enzyme.

Comparative studies on molecular weights of proteins are often narrow in scope because the techniques available are tedious and/or require relatively large amounts of highly purified material. The advent of gel filtration has provided a simple, rapid, and relatively accurate means of determining relative molecular sizes of proteins with molecular weights as high as 300,000.

Granath and Flodin (1961) demonstrated an excellent correlation between the molecular weight of a series of dextrans and their gel filtration elution pattern. Further investigations by Whitaker (1963) and Andrews (1964, 1965) showed that gel filtration could also be used for the estimation of molecular weights of proteins; however, more recent studies (Ackers, 1964; Siegel and Monty, 1966) indicate that the elution volume of a protein is a function of its molecular size expressed as the Stokes (molecular) radius.

We have used the gel filtration technique to survey malate dehydrogenases in both purified crystalline preparations and in crude cell-free extracts. Our survey indicates that the malate dehydrogenases of most animals, plants, and microorganisms are of equal molecular size, but in certain bacteria these enzymes are significantly larger.

Materials and Methods

Sephadex G-100, G-150, and G-200 and Dextran Blue were purchased from Pharmacia Fine Chemicals; horse heart cytochrome *c* from the Sigma Biochemical Co.; oxaloacetic and L-malic acids from Nutritional Biochemicals Corp.; nitro blue tetrazolium and phenazine methosulfate from Mann Research Laboratories; chicken ovalbumin from Pentex Inc.; DPNH[1] from

* From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts. *Received June 22, 1966.* Publication No. 472. This study was supported in part by grants from the National Institutes of Health (CA-03611), National Aeronautics and Space Administration (NGS-375), and the American Cancer Society (P-77H, the Charles Simon Memorial Grant for cancer research, and American Cancer Society Institutional Grant 1N29).

† Postdoctoral Fellow of the National Institute of Child Health and Human Development, F2 HD-8683902. Present address: Children's Hospital, State University of New York at Buffalo, Buffalo, N. Y.

‡ Durkee Graduate Fellow of the Glidden Co. Present address: Clayton Foundation Biochemical Institute, Department of Chemistry, University of Texas, Austin, Texas.

[1] Abbreviations used: DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; MDH, malate dehydrogenase.