ENZYMATIC SYNTHESIS OF CELL WALL MUCOPEPTIDE IN A PARTICULATE PREPARATION OF ESCHERICHIA COLI

Y. Araki, R. Shirai, A. Shimada, N. Ishimoto and E. Ito

Department of Chemistry, Faculty of Science,
University of Hokkaido, Sapporo, Japan

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In cell wall mucopeptide of Staphylococcus aureus, backbone polysaccharide chains are covalently linked through peptide bridges containing pentaglycine. Enzymatic synthesis of a mucopeptide precursor from UDP-GlcNAc[1], UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala and glycine has been demonstrated in a cell-free system of S. aureus (Chatterjee et al., 1964; Meadow et al., 1964; Anderson et al., 1965). This enzymatic product was devoid of the cross-linkage and contained two D-alanine residues originating in terminal D-Ala-D-Ala residue of the substrate, while the cell wall mucopeptide from S. aureus contains one D-alanine residue per D-glutamic acid residue (Anderson et al., 1965).

In Escherichia coli cell wall mucopeptide, each muramic acid residue is linked to the terminal amino group of a peptide L-Ala-D-Glu-meso-DAP-D-Ala. Some terminal carboxyl group of this peptide is cross-linked to an amino group of diaminopimelic acid of a neighboring peptide (Weidel et al., 1964). The present

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[1] The abbreviations used are: GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; DAP, diaminopimelic acid.

communication describes the enzymatic synthesis of cross-linked mucopeptide from UDP-MurNAc-L-Ala-D-Glu-meso-DAP-D-Ala-D-Ala and UDP-GlcNAc by a particulate preparation of E. coli.

Materials and Methods: The substrates labeled in the amino acid were enzymatically prepared using crude enzymes from S. aureus, E. coli and Bacillus cereus as described previously (Ito et al., 1962). Cells of E. coli B harvested at 40 % maximum growth were disrupted in 0.02 M potassium phosphate, pH 7.8, 0.001 M EDTA by a 10 Kc sonic oscillator for 7 min, and the particulate fraction was sedimented between 20,000 x g and 105,000 x g, washed twice in 0.02 M Tris-HCl, pH 7.8, and was resuspended in 0.02 M Tris-HCl, pH 7.2.

Incubation was carried out in the reaction mixture described in Table 2 for 60 min at 37°. After heating for 2 min in a boiling water bath, the reaction mixture was treated with 0.3 M perchloric acid and the resulting precipitate, after washing twice in 0.3 M perchloric acid and once in 80 % ethanol, was assayed for radioactivity in a gas flow counter.

Preparation and characterization of lysozyme fragmentation products from cell wall mucopeptide were carried out as described by Primosigh et al., (1961).

Results and Discussion: As seen in Table 1, UDP-MurNAc-L-Ala-D-Glu-meso-DAP-D-Ala-D-Ala and to a lesser extent UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala were utilized as substrate for the synthesis of mucopeptide in the E. coli particulate system. The activity toward both UDP-MurNAc-pentapeptides suggests that the presence of a specific enzyme which adds meso-diaminopimelic acid to UDP-MurNAc-L-Ala-D-Glu is the basis for the occurrence of this amino acid in E. coli cell wall mucopeptide (Strominger

Table 1. Incorporation of radioactivity from UDP-MurNAc-peptides into mucopeptide.

Substrates	Incorporation, cpm
UDP-MurNAc-Ala-^{14}C-Glu	30
UDP-MurNAc-Ala-^{14}C-Glu-Lys	15
UDP-MurNAc-Ala-^{14}C-Glu-DAP	5
UDP-MurNAc-Ala-^{14}C-Glu-Lys-Ala-Ala	295
UDP-MurNAc-Ala-^{14}C-Glu-DAP-Ala-Ala	680

The reaction mixture contained 38 mμmoles (36,000 cpm) of UDP-MurNAc-peptides, 40 mμmoles of UDP-GlcNAc, 0.96 mg of cell particles and other components in a total volume of 100 μl. The values were subtracted with boiled enzyme control values.

Table 2. Requirement for reaction mixture.

Reaction mixture	Incorporation, cpm
Complete	780
Boiled enzyme control	30
- UDP-GlcNAc	35
UDP-MurNAc-pentapeptide hydrolyzed	30
- UDP-GlcNAc + UDP-MurNAc, 10 mμmoles	65
+ ATP, 25 mμmoles	1,360
- $MgCl_2$	30

The complete reaction mixture contained 5.72 mμmoles (13,000 cpm) of UDP-MurNAc-L-Ala-D-Glu-meso-DAP-^{14}C-D-Ala-^{14}C-D-Ala, 10 mμmoles of UDP-GlcNAc, 4 μmoles of $MgCl_2$, 4 μmoles of Tris-HCl, pH 7.2 and 0.9 mg of particles in a final volume of 100 μl. UDP-MurNAc-pentapeptide hydrolyzed in 0.01 N HCl for 15 min at 100° was used as indicated.

et al., 1961). Requirement for UDP-GlcNAc and $MgCl_2$ and stimulation by ATP were indicated (Table 2). Addition of amino

acids, peptides, oxamycin and chloramphenicol had no influence. The reaction product was insoluble in water, 0.1 N LiOH and organic solvents. Neither boiling in 0.01 N HCl for 15 min nor treatment with trypsin and pepsin could release the incorporated radioactivity. Incubation of the reaction mixture with lysozyme resulted in disappearance of radioactivity from the mucopeptide fraction.

In order to obtain reference samples of mucopeptide fragments, cell walls of E. coli B, after treatment with 0.4 % Na-dodecyl-sulfate, were digested with excess lysozyme and the main fragmentation products C3, C4 and C6 were separated by ethanol fractionation followed by repeated paper chromatography in several solvents. Hydrolysis of these fragments for 12 hours at 95° in 4 N HCl gave a mixture of glucosamine, muramic acid, glutamic acid, diaminopimelic acid and alanine. Determination of molar ratios with an amino acid autoanalyzer demonstrated a proportion of 1:1:1:1:2 (alanine) for the five components of these fragments. Dinitrophenylation of C3, C4 and C6 followed by hydrolysis and paper chromatography gave 0.50, 0.51 and 0.95 mole of monodinitrophenyl-DAP and 0.52, 0.47 and 0.03 mole of free diaminopimelic acid per mole of glutamic acid, respectively. Reduction of the fragments with $NaBH_4$ resulted in disappearance of all muramic acid residues from C3 and C6, and of more than half of muramic acid residues from C4. Determination of reducing group by the method of Park et al. (1949) with heating time of 5 min using glucose as standard, demonstrated 1.05, 0.60 and 1.03 moles of reducing end per mole of glutamic acid for C3, C4 and C6, respectively. Treatment of C3 and C6 with β-glucosaminidase resulted in liberation of all glucosamine residues, yielding MurNAc-peptide (from C6) and another product presumed

as a dimer of MurNAc-peptide (from C3) in addition to N-acetyl-glucosamine (Fig. 1). The above results, together with other evidence e.g. molecular weights described by Primosigh et al., are consistent with the structures (GlcNAc-MurNAc-Ala-Glu-DAP-Ala)$_2$ for C3 and C4, and GlcNAc-MurNAc-Ala-Glu-DAP-Ala for C6 as proposed by Weidel et al. (1964). In C3 and C4, a carboxyl group of one peptide unit is bound to an amino group of diaminopimelic acid of the other peptide unit linking two disaccharide units. In C4, disaccharide units are further linked through a glycosidic bond. Though it remains to be seen what carboxyl

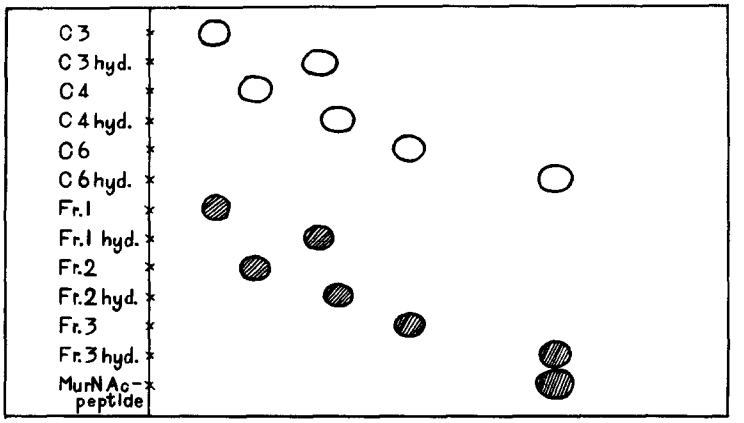

Fig. 1. Paper chromatogram showing identification of fragments from lysozyme digestion of enzymatic product with those from lysozyme digestion of cell wall mucopeptide. 27 mg of E. coli particles were incubated for 60 min with 152 mμmoles (660,000 cpm) of UDP-MurNAc-pentapeptide labeled in D-alanine, 500 mμmoles of UDP-GlcNAc, 120 μmoles of $MgCl_2$, 100 μmoles of Tris-HCl, pH 7.2 in a final volume of 2.8 ml. Mucopeptide fraction separated by precipitating in 0.3 M perchloric acid and washing in 80 % ethanol was digested with 7 mg of lysozyme in 2 ml of 0.05 M Tris-HCl, pH 7.2 for 2 days at 37°. The digest was separated by paper chromatography in butanol-acetic acid-water (4:1:5) for 3 days into three fractions (Fractions 1, 2 and 3). Samples and their β-glucosaminidase hydrolysates were subjected to paper chromatography with reference samples from E. coli cell wall mucopeptide (C3 and C4, cross-linked tetrasaccharides; C6, disaccharide) in butanol-acetic acid-water (4:1:5) for 3 days. β-glucosaminidase was prepared from pig epididymis (Findlay et al., 1960). The fragments from the enzymatic product were detected by radioautography and the reference samples were by ninhydrin reaction.

group is involved in the cross-link, the formation of these fragments on lysozyme digestion has provided evidence for the occurrence of an inter- or intra-polysaccharide peptide bridge in the cell wall mucopeptide (Weidel et al., 1964).

Digestion with excess lysozyme was carried out on a large-scale preparation of mucopeptide fraction synthesized from the substrate labeled in D-alanine. Radioactive fragments, Fractions 1, 2 and 3 separated by paper chromatography were coincident with the reference samples C3, C4 and C6 respectively, on paper chromatograms in butanol-acetic acid-water (4:1:5) (Fig. 1), 80 % ethanol, isobutyric acid-0.5 N NH_3 (1:0.6) and butanol-pyridine-acetic acid-water (60:40:3:30). On hydrolysis with β-glucosaminidase, moreover, each radioactive fragment gave a product which was coincident with the ninhydrin-positive product from the corresponding reference sample.

More direct evidence for the occurrence of the cross linkage in the enzymatic product was obtained starting with the substrate labeled in diaminopimelic acid (Table 3). After dinitrophenylation followed by hydrolysis, half of diaminopimelic acid in the tetrasaccharides was recovered as free amino acid indicating that diaminopimelic acid in one peptide unit is involved in the cross-linkage.

Another characteristic feature of the enzymatic product in the E. coli particulate preparation was the low content of D-alanine. Analysis of the reaction product from the substrate labeled in both glutamic acid and D-alanine indicated that the product contained 1.2 moles of D-alanine per mole of glutamic acid.

Although determination of nucleotides released from the substrates in transglycosylation was unsuccessful because of

Table 3. Dinitrophenylation of fragments from enzymatic product

Fragments	Diaminopimelic acid	Mono-dinitrophenyl-diaminopimelic acid
	μμmoles	μμmoles
Tetrasaccharides	108	111
Disaccharide	7	108

Synthesis of mucopeptide from 15 mμmoles (50,000 cpm) of UDP-MurNAc-pentapeptide labeled in diaminopimelic acid, lysozyme digestion of the product, and separation of the fragments were carried out as described in Fig. 1. Tetrasaccharide fraction including Fractions 1 and 2, and disaccharide fraction (Fraction 3) were eluted from the paper and subjected to treatment with fluorodinitrobenzene followed by hydrolysis and paper chromatography according to the method of Primosigh et al. (1961). 20 mμmoles of MurNAc-Ala-Glu-DAP were added as a carrier at dinitrophenylation. Spots of diaminopimelic acid and its derivative were extracted and assayed for radioactivity.

a marked pyrophosphatase activity occurring in the particulate preparation, the present evidence suggests that in the Gram-negative bacteria mucopeptide is synthesized essentially in the same mechanism as that in the Gram-positive one.

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