EFFECT OF PENICILLIN ON CELL WALL MUCOPEPTIDE SYNTHESIS IN A ESCHERICHIA COLI PARTICULATE SYSTEM

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Wise et al. (1965) observed using Staphylococcus aureus cells that mucopeptide formed in the presence of penicillin contained excess of D-alanine and terminal amino group of glycine. From this evidence they suggested that at a final stage of mucopeptide synthesis a penicillin-sensitive transpeptidation reaction links inner D-alanine to the free amino group of a neighboring peptide chain with the release of terminal D-alanine, thus joining neighboring polysaccharide chains. The proposed mechanism remains to be confirmed since significant influence of penicillin on mucopeptide synthesis has not been demonstrated in the S. aureus cell-free system (Chatterjee et al., 1964; Meadow et al., 1964; and Anderson et al., 1965). The incorporation of two D-alanine residues per repeating unit into the enzymatic product suggests that the transpeptidation system was not working in the cell-free system of S. aureus.

The present authors have studied enzymatic synthesis of mucopeptide in a particulate preparation of E. coli (Araki et al., 1965). The enzymatic product contained the peptide cross-linkage (Araki et al., 1966). Therefore, using this system, it was anticipated that the formation of cross-linkages would be influ-

enced by penicillin. Evidence described in the present communication demonstrates: (1) that 50 units/ml of penicillin completely interferes with the formation of cross-linked mucopeptide in the E. coli particulate system; (2) that penicillin inhibits the liberation of D-alanine from the precursor and the intermediates; and (3) that soluble products are formed in the presence of penicillin.

Materials and Methods: The preparation of the substrates labeled in amino acids, the preparation of the E. coli particulate fraction and the incubation for mucopeptide synthesis were carried out as described elsewhere (Araki et al., 1966). The reaction product separated either by paper chromatography in isobutyric acid-0.5 M NH_3 (1:0.6) or by precipitating and washing in 0.3 M perchloric acid was assayed for radioactivity in a gas flow counter.

Results and Discussion: Mucopeptide synthesis as assayed in either method was rather enhanced by penicillin. When the reaction product formed in the presence of penicillin was precipitated with perchloric acid and then extracted with 80 % ethanol, a larger portion of its radioactivity was transferred into the ethanol supernatant. As seen in Fig. 1, in the presence of penicillin the ethanol soluble fraction increased with time, while in the control experiment this fraction occurred in a low steady level. This result suggests that penicillin interferes with the conversion of an ethanol soluble intermediate into insoluble mucopeptide. The products formed under the influence of penicillin stayed at the origin of paper chromatogram and were converted from a water insoluble form into a water soluble form by heating in 0.01 N HCl for 15 min at 100°.

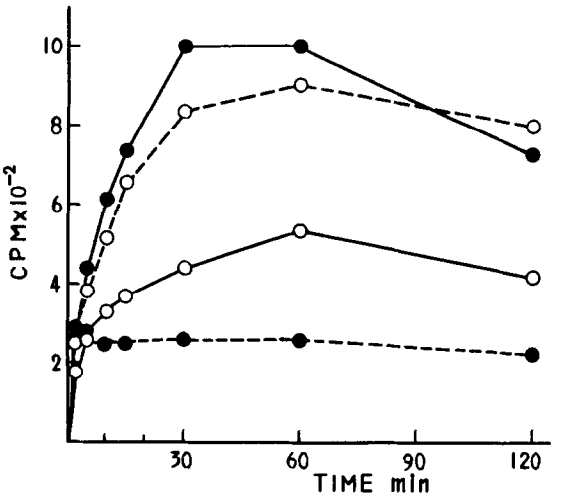

Fig. 1. Time course of formation of ethanol soluble and insoluble fractions. Reaction mixture containing 6 mμmoles (13,000 cpm) of UDP-MurNAc-Ala-Glu-DAP-^{14}C-Ala-^{14}C-Ala [1], 20 mμmoles of UDP-GlcNAc, 0.96 mg of the particles and other components with (○) or without (●) 5 units of penicillin G in a final volume of 100 μl was incubated for the indicated time at 37°. The mucopeptide fraction precipitated in 0.3 M perchloric acid was extracted with 0.8 ml of 80 % ethanol. The precipitate (——) and the supernatant (----) were separated and assayed for radioactivity.

The ethanol insoluble fraction formed in the absence of penicillin gave a considerable amount of tetrasaccharides on digestion with lysozyme, providing evidence for the occurrence of the peptide cross-linkage in the enzymatic product. Identification of these tetrasaccharides with the cross-linked tetrasaccharides obtained by lysozyme fragmentation of E. coli cell wall mucopeptide was described elsewhere (Araki et al., 1966). On the other hand, the product formed under the influence of penicillin was almost completely hydrolyzed into disaccharide (Table 1). The peptide cross-linkage in E. coli mucopeptide

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

Table 1. Lysozyme digestion of mucopeptide fraction.

Incubation	Ethanol insoluble fraction		Fragments from lysozyme digestion			
			Tetrasaccharides		Disaccharide	
	Yield	D-Ala/Glu	Yield	D-Ala/Glu	Yield	D-Ala/Glu
	cpm	mole/mole	cpm	mole/mole	cpm	mole/mole
Control	20,300	1.15	7,200	0.90	8,900	1.27
Penicillin	10,500	1.85	400	-	9,400	1.88

Mucopeptide was synthesized in a reaction mixture containing 100 mμmoles (750,000 cpm) of UDP-MurNAc-Ala-^{14}C-Glu-DAP-^{14}C-Ala-^{14}C-Ala (prepared enzymatically using glutamic acid and alanine having approximately equal specific activities), 1 μmole of UDP-GlcNAc, and 45 mg of the particles in 5.5 ml of 0.04 M $MgCl_2$, 0.04 M Tris-HCl, pH 7.2 with or without 250 units of penicillin G for 60 min at 37°. The reaction mixture was treated in 0.3 M perchloric acid and the precipitate, after washing in 80 % ethanol, was digested with 6 mg of lysozyme in 4.4 ml of 0.02 M Tris-HCl, pH 7.2 for 48 hours at 37°. Fragments from the digestion were separated by paper chromatography in butanol-acetic acid-water (4:1:5) for 3 days. Samples of mucopeptide fraction and its fragments were hydrolyzed in 6 N HCl for 12 hours at 95°, and amino acids separated by paper chromatography in butanol-pyridine-acetic acid-water (60:40:3:30) were eluted and assayed by radioactivity measurement.

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should involve an amino group of diaminopimelic acid. Determination of free amino group confirmed that one amino group of diaminopimelic acid in the mucopeptide formed under the influence of penicillin was almost totally free. These results indicate that penicillin interferes with the peptide cross-linkage formation (Table 2).

Moreover, penicillin inhibited the elimination of D-alanine (Table 1). The product formed under the influence of penicillin contained two D-alanines per glutamic acid while tetrasaccharides from the control experiment contained one D-alanine per glutamic acid. This observation strongly supports the mechanism that

Table 2. Dinitrophenylation of diaminopimelic acid incorporated.

Incubation for incorporation	Recovery after dinitrophenylation	
	Free DAP	Mono-DNP-DAP
	μμmoles	μμmoles
Control	63	190
Penicillin	4	108

Incubation for mucopeptide synthesis was carried out for 1 hour at 37° in a reaction mixture containing 12 mμmoles (40,000 cpm) of UDP-MurNAc-Ala-Glu-^{14}C-DAP-Ala-Ala, 40 mμmoles of UDP-GlcNAc, 2 mg of the particles and other components with or without 10 units of penicillin in a final volume of 200 μl. Mucopeptide fraction separated by perchloric acid precipitation and ethanol treatment was subjected to treatment with FDNB 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 on dinitrophenylation. Spots of diaminopimelic acid and its derivative were assayed for radioactivity after elution.

penicillin interferes with cross-linking transpeptidation, in which with release of terminal D-alanine, the carboxyl group of inner D-alanine of a peptide unit is linked to the amino group of diaminopimelic acid of a neighboring peptide unit.

However, the elimination of D-alanine is not always correlated to the transpeptidation, since the disaccharide from the control experiment contained less than two D-alanine residues. Actually, the disaccharide fraction was found to be a mixture. MurNAc-Ala-Glu-DAP-Ala and MurNAc-Ala-Glu-DAP-Ala-Ala were obtained from the dissacharide fraction after its β-glucosaminidase treatment followed by paper chromatography (Table 3). The two compounds were identified as those by comparing with reference samples on paper chromatograms and from their content of D-alanine per glutamic acid. The above evidence suggests that some D-alanine

Table 3. Separation of MurNAc-Ala-Glu-DAP-Ala and MurNAc-Ala-Glu-DAP-Ala-Ala from hydrolysate of disaccharide.

Fraction	Rf value	Alanine	Glutamic acid	Molar ratio Ala/Glu
		μμmoles	μμmoles	
Spot 1 (MurNAc-tetrapeptide)	0.28	585	545	1.07
Spot 2 (MurNAc-pentapeptide)	0.32	530	285	1.86

Synthesis of mucopeptide from UDP-MurNAc-Ala-^{14}C-Glu-DAP-^{14}C-Ala-^{14}C-Ala in absence of penicillin, lysozyme degradation and separation of disaccharide were carried out as described in Table 1. The disaccharide fraction was hydrolyzed with excess pig epididymis β-glucosaminidase. The resulting radioactive monosaccharide separated by paper chromatography in butanol-acetic acid-water (4:1:5) as one spot, was rechromatographed in phenol-water containing 0.04 % 8-hydroxyquinolin (4:1) giving two spots (Spots 1 and 2) with Rf values indicated. Each spot was eluted and analyzed for amino acid by radioactivity measurement after hydrolysis followed by paper chromatography and elution.

Table 4. Mucopeptide synthesis from UDP-MurNAc-tetrapeptide.

Substrate	Incorporation	
	cpm	μμmoles*
UDP-MurNAc-pentapeptide	1,060	220
UDP-MurNAc-tetrapeptide	200	42

* The values were calculated assuming that the product contained one D-alanine per glutamic acid.

2.2 mμmoles (16,000 cpm) of UDP-MurNAc-Ala-^{14}C-Glu-DAP-^{14}C-Ala-^{14}C-Ala or 2.5 mμmoles (12,000 cpm) of UDP-MurNAc-Ala-^{14}C-Glu-DAP-^{14}C-Ala was incubated for 1 hour at 37° with 10 mμmoles of UDP-GlcNAc, 0.48 mg of the particles and other components in a final volume of 50 μl. The mucopeptide fraction precipitated in 0.3 M perchloric acid and washed in 80 % ethanol was assayed for radioactivity. D-alanine and glutamic acid in the substrates had approximately equal values of specific radioactivity.

Table 5. Effect of penicillin on D-alanine liberation.

Addition	D-alanine liberation
	cpm
None	5,065
Penicillin	360

Reaction mixture containing 6 mμmoles (13,000 cpm) of UDP-MurNAc-Ala-Glu-DAP-^{14}C-Ala-^{14}C-Ala, 10 mμmoles of UDP-GlcNAc, 0.96 mg of the particles, 4 μmoles of Tris-HCl, pH 7.2 and 4 μmoles of $MgCl_2$ with or without 5 units of penicillin in a final volume of 100 μl was incubated for 60 min at 37°, and then subjected to paper chromatography in isobutyric acid-0.5 M NH_3 (1:0.6). Spot of alanine was eluted and assayed for radioactivity.

is released from mucopeptide hydrolytically and this hydrolysis also is inhibited by penicillin.

The particulate preparation of E. coli was found to have considerable activity of D-alanine peptidase, which split D-Ala-D-Ala linkage of UDP-MurNAc-pentapeptide but was inactive toward free D-Ala-D-Ala. From the reaction mixture after mucopeptide synthesis, the excess substrate was recovered as UDP-MurNAc-Ala-Glu-DAP-Ala and free D-alanine. Therefore, one may suspect that UDP-MurNAc-tetrapeptide rather than UDP-MurNAc-pentapeptide is the immediate precursor for mucopeptide synthesis. However, this seems unlikely since mucopeptide synthesis using UDP-MurNAc-tetrapeptide as substrate demonstrated this compound to be only 20 % as active as UDP-MurNAc-pentapeptide (Table 4).

The above evidence indicates that with the particulate preparation of E. coli, terminal D-alanine is released at least in the three reactions: hydrolysis of the substrate itself, cross-linking transpeptidation, and hydrolysis of mucopeptide. Although it is not known whether or not these peptidase and trans-

peptidase activities are due to a single enzyme, all these reactions proved to be inhibited by penicillin. As seen in Table 5, overall liberation of D-alanine was almost completely suppressed by penicillin. Thus, penicillin is now understood as an inhibitor of peptidase and transpeptidase specific for terminal D-Ala-D-Ala linkage. β-lactam ring of penicillin has been proposed as the site essential for its inhibitory activity on the basis of its structural resemblance to the mucopeptide intermdeiate (Wise et al., 1965; and Tipper et al., 1965).

The present result strongly supports the suggestion by Wise et al. that inhibition of the cross-linking transpeptidation is ultimate cause of the bactericidal action of penicillin. The alternative mechanism for the cross-linkage formation, that is, cross-linkage synthesis following hydrolytic elimination of D-alanine seems unlikely since the cross-linkage formation in the present system did not require ATP which is usually essential in de novo synthesis of a peptide linkage.

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