MICROCOCCUS LYSODEIKTICUS: A NEW TYPE OF CROSS-LINKAGE OF THE MUREIN

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In the past six years the cell wall of Micrococcus lysodeikticus has been extensively investigated (5, 7, 8, 10, 11 and 15), but the mode of cross-linkages of the pentapeptide subunits has remained obscure. The most striking result has been that 40 - 50 % of the muramic acid of the glycan is unsubstituted by peptides (8), whereas the number of pentapeptide subunits of the cell wall is approximately equal to the number of disaccharide units (7).

The present work shows that the cross-linkage also are formed by the pentapeptide, which is linked by the C-terminal D-alanine to the ε-aminogroup of lysine and by the N-terminal L-alanine to the carboxylgroup of the C-terminal D-alanine of an adjacent pentapeptide.

Methods and results:

Micrococcus lysodeikticus (ATCC 12 698) was grown in broth (1 % peptone from casein, 0,5 % yeast extract, 1 % glucose, pH = 7,0 - 7,2) at 30° C under aerobic conditions (shaker) and harvested in the stationary phase. Cell walls were prepared by the usual technique (4). The cells were disintegrated in a cell mill with glass beads and the cell walls harvested by centrifugation. Further purification was achieved by incubation with trypsin for 12 hrs and extraction with trichloroacetic acid.

Paper chromatography was carried out on SS 2043b using the following solvent systems:

A Isopropanol/acetic acid/water = 75 : 10 : 15

B α-Picoline/conc. NH_4OH/water = 70 : 2 : 28

C n-Butanol/pyridine/acetic acid/water = 42 : 28 : 2,1 : 21

The configuration of alanine was determined enzymatically (3, 16).

Composition of the cell wall:

The TCA-extracted cell walls of M. lysodeikticus contained muramic acid, glucosamine, glutamic acid, lysine, glycine and alanine approximately at a molar ratio of 1:1:1:1:1:2 (Table 1).

The determination of the configuration of alanine yielded equimolar amounts of L- and D-alanine.

Table 1: Amino acid and amino sugar composition of cell walls of M. lysodeikticus (Amino acid analyzer; Bender & Hobein, Munich)

Components	µmole/mg	ratio (glu = 1,00)
glucosamine	0,78	1,13
muramic acid	0,594	0,96
alanine	1,27	1,84
glycine	0,72	1,04
glutamic acid	0,69	1,00
lysine	0,63	0,92
ammonia	0,33	0,48

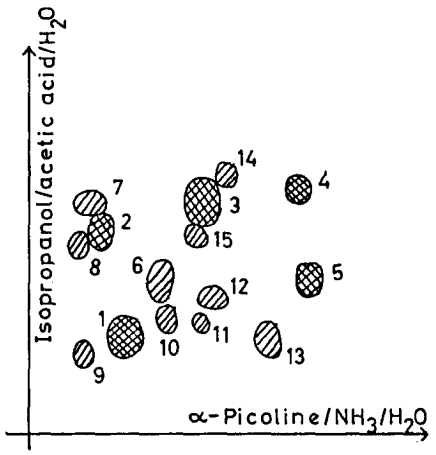
Determination of the free amino groups of the murein: TCA-extracted cell walls were dinitrophenylated and hydrolyzed (4N HCl, 100° C, 16 hrs). The analysis of the hydrolysate by paper chromatography (1,5 M phosphate buffer pH = 6,0; n-propanol/0,2 % NH_4OH = 8 : 2) showed ε-DNP-lysine and traces of DNP-alanine. Approximately 30 % of lysine was found to be dinitrophenylable. Katz and Strominger (1967) found 32 % ε-DNP-lysine and 3 % DNP-alanine. How-

ever, since the dinitrophenylation method generally yields too low values, the amount of free ε-amino groups of lysine is probably 40 - 50 %.

Determination of the amino acid sequence:

TCA-extracted cell walls were partially hydrolysed (100° C, 4N HCl, 1 hour). Fig. 1 shows a typical two dimensional paper chromatogram of a partial hydrolysate.

Fig. 1: Chromatogram of partial hydrolysate of M. lysodeikticus cell walls (1 = Lys, 2 = Glu, 3 = Ala, 4 = Mur, 5 = $GlcNH_2$, 6 = Gly, 7 = Ala-Glu, 8 = Glu-Gly, 9 = Glu-Lys, 10 = Lys-D-Ala, 11 = N^6-D-Ala-Lys, 12 = N^6-D-Ala-Lys-D-Ala, 13 = Mur-$GlcNH_2$, 14 = D-Ala-L-Ala, 15 = unidentified pink spot)



The various peptides were separated and isolated by repeated one dimensional paper chromatography using solvent systems A, B and C. The peptides were identified by determining the molar ratio of the amino acids, the N-terminal amino acid and the configuration of alanine. The occurence of the peptides L-Ala-Glu, Glu-Gly, Glu-Lys and Lys-D-Ala confirmed the amino acid sequence of the pentapeptide (L-Ala-D-Glu(Gly)-Lys-D-Ala) as found by Mirleman and Sharon (1966) and by Katz and Strominger (1967).

A further ninhydrin positive spot (Nr. 13) yielded equimolar amounts of muramic acid and glucosamine. The appearance of this disaccharide is in agreement with the findings of Ley-Bouille et al. (1966) and of Munoz et al. (1966a), that a significant portion of the muramic acid is not substituted by a pentapeptide. The disaccharide has never been found in partial hydrolysates of other mureins, which contain no unsubstituted muramic acid, since the glycoside bond is less resistent to acid hydrolysis than the peptide bond between muramic acid and L-alanine.

The 3 other peptides (Nr. 11, 12 and 14) are important for the elucidation of the cross-linking. Peptide Nr. 11 is the dipeptide N^6-D-Ala-L-Lys and the neighbour peptide Nr. 12, the tripeptide N^6-D-Ala-L-Lys-D-Ala. They show that the cross-linking peptide is bound to the ε-amino group of lysine by its C-terminal D-alanine.

Peptide Nr. 14 yielded D-alanine and L-alanine in equimolar amounts and cochromatographed in the solvent systems A and B with authentic D-Ala-L-Ala*. It was clearly separated from L-Ala-L-Ala or D-Ala-D-Ala, respectively.

Such a peptide would not be found during hydrolysis, if the mode of cross-linking as suggested by Katz and Strominger (1967), were correct. Its occurrence indicates that the cross-linking peptide is bound to the C-terminal D-alanine of a pentapeptide by its N-terminal L-alanine. In other mureins, where alanine is involved in the cross-linking, more than 2 moles of alanine per mole of glutamic acid are present (6, 13, 16, 17). The additional alanine shows L-configuration. The murein of M. lysodeikticus, however, contains no additional alanine and D-alanine is bound to the

*All authentic alanine peptides were kindly supplied by Prof. F. Weygand, Institut für Organische Chemie, Technische Hochschule München

ε-aminogroup of lysine instead of L-alanine. The partial hydrolysates yielded only peptides, which are compatible with the amino acid sequence of the pentapeptide, but no additional peptides, suggesting a cross-linking peptide of another amino acid sequence. It is likely, therefore, that the cross-linking peptide is identical with the pentapeptide. Fig. 2 shows the structure of the murein, which is compatible with our own data as well as those of other authors (7, 6, 12).

A similar structure has been proposed by Pickering (1966) who compiled data from other authors but had no direct evidence. In contrast with his scheme the structure shown in Fig. 2 takes into consideration that half of the muramic acid is unsubstituted by a pentapeptide, while the ratio of disaccharide moieties and pentapeptides is still 1 : 1.

The biosynthesis of the murein of M. lysodeikticus has been extensively studied (1, 2, 9). As pointed out by Strominger and Katz (1967) there is no evidence for the incorporation of disaccharides with an unsubstituted muramic acid. Therefore, we

Fig. 2: Amino acid sequence of a part of the peptidoglycan of M. lysodeikticus. Only intra-chain bridges are shown, although both, intra- and inter-chain bridges certainly occur.

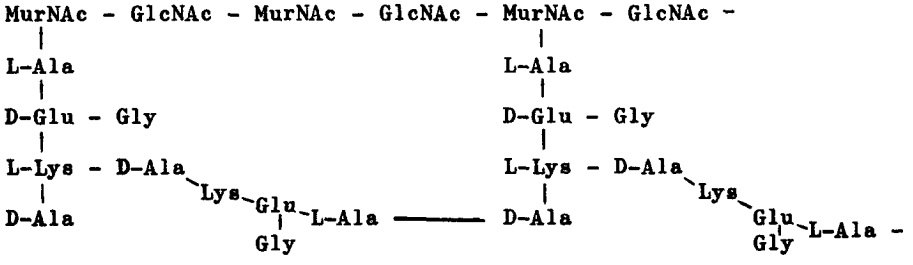
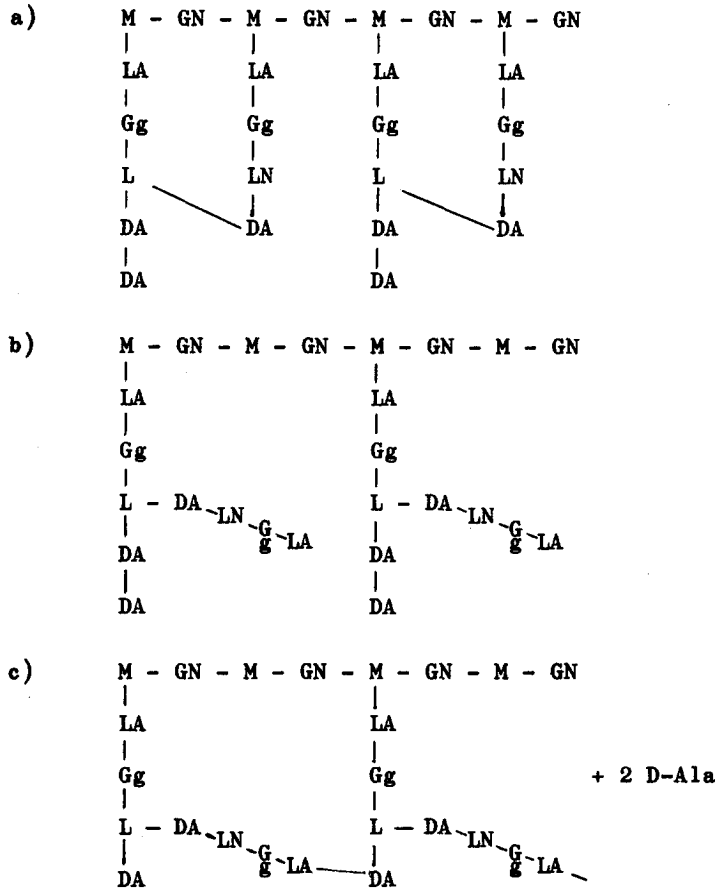


Fig. 3: Scheme for the hypothetical biosynthesis of the cross-linkage of peptidoglycan of M. lysodeikticus (Abbreviations: M = muramic acid, GN = glucosamine, LA = L-alanine, G = glutamic acid, g = glycine, L = lysine, DA = D-alanine. LN = lysine with free ε-amino group)



suggest that the biosynthetis of the murein occurs in the usual manner, forming a glycan substituted by hexapeptides which contain two D-alanine at the C-terminal end. As indicated in Fig. 3a the penultimate D-alanine of a hexapeptide is bound to the ε-amino-group of the lysine of an adjacent hexapeptide by transpeptidation as proposed by Wise and Park (1965). Then, the pentapeptide is

released from the muramic acid by muramyl-L-alanine amidase (Fig. 3b) which is known to occur in E.coli (18) and in B.subtilis (20). The N-terminal L-alanine is then bound to the penultimate D-alanine of an adjacent hexapeptide by the fore-mentioned transpeptidation (Fig. 3c). This sequence of reactions would explain the biosynthesis of the structure shown in Fig. 2. According to this suggestion one has only to assume the participation of muramyl-L-alanine amidase - so far regarded as a degrading enzyme - in a synthetic step.

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