PEPTIDE CROSSLINKAGE IN CELL WALL MUREIN OF PROTEUS MIRABILIS AND ITS PENICILLIN-INDUCED UNSTABLE L-FORM

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Summary: In mureins of normal rod-shaped P.mirabilis and its penicillin-induced unstable L-form approximately 33 % of the diaminopimelic acid residues participate in peptide crosslinkage. No decrease of crosslinkage due to an effect of penicillin was observed.

Recent research on the mode of action of penicillin has been concerned with the interference of the antibiotic with the crosslinkage of peptide side chains in the murein (peptidoglycan) of bacterial cell walls. In cell-free extracts of Escherichia coli crosslink formation was shown to operate by transpeptidation. The components of the reaction are the pentapeptide side-chains (-NH-L-Ala-D-isoGlu-m-Dap-D-Ala-D-Ala-COOH)* of two muropeptide subunits which have been freshly incorporated into the glycan backbone of the polymer. The side-chains are connected by peptide bond formation between the penultimate D-Ala of one chain and the free amino group of m-Dap on the other chain. At the same time C-terminal D-Ala is released from the pentapeptides either as a step in the transpeptidation reaction itself or by separate action of a D-Ala-carboxypeptidase (1). In the same in vitro system Izaki et al. (1) and Araki et al. (2) have demonstrated an inhibition of the crosslinking transpeptidase and the D-Ala-carboxypeptidase during murein biosynthesis in the presence of penicillin. As a consequence linear peptidoglycan with uncrosslinked pentapeptide side-chains was formed.

Analysis of the murein of the unstable L-form of Proteus mirabilis synthesized in vivo in penicillin medium yielded different results. Inhibition of alanine release and formation of murein containing pentapeptide side-chains could not be found (3,4). Earlier chemical end-group determinations seemed to indicate a lesser degree of peptide crosslinkage in L-form murein than in normal murein of

*Abbreviations: Ala = alanine; Glu = glutamic acid; isoGlu = isoglutamyl; Dap = diaminopimelic acid; Mur = muramic acid; GlcN = glucosamine; DNP = dinitrophenyl; FDNB = 1-fluoro-2,4-dinitrobenzene.

P.mirabilis (4). Reinvestigation of this problem has not confirmed these results. Data presented in this report reveal that normal Proteus murein and murein formed in the presence of penicillin differ very little, if at all, in their peptide crosslinkage.

METHODS:

Proteus bacteria and L-forms were grown in the following medium: Tryptic casein peptone (E.Merck, Darmstadt, Germany) 15 g; beef extract Lab-Lemco (Oxoid) 15 g; glucose 2 g; NaCl 5 g; Na_2HPO_4 4.5 g; distilled water 1000 ml, pH 7.2. L-form cultures also contained 200 units penicillin G per ml.

Mass production of cells was carried out in 10 liter batches of vigorously aerated liquid medium at 35°C. Incubation was stopped at the onset of the stationary phase by instant cooling of the culture in an ice bath and immediately harvesting the cells in a cooled continuous flow centrifuge.

Preparation of empty cell walls from bacteria and L-forms has been described (5). All steps of the procedure were performed either at low temperature or in the presence of at least 0.8 % (w/v) sodium dodecylsulfate (SDS) in order to preclude autolysis of murein. Murein was isolated by extracting cell walls twice with 4 % (w/v) SDS for 15 min at 100°C, sedimenting crude murein sacculi by centrifugation for 60 min at 25 000 g and liberating them from covalently attached protein by digestion with pancreatin (E.Merck, Darmstadt, Germany; 100 mg pancreatin/ml in 0.066 M phosphate buffer, pH 7.0, 15 hrs. incubation at 37°C).

Reaction of free amino groups with fluorodinitrobenzene was carried out as follows: 0.5 to 1.0 µmoles of murein (based on glutamic acid equivalents) in a 4 % (v/v) solution of triethylamine in water : ethanol (10:1, v/v) were reacted with 1.0 ml of a 5 % (v/v) ethanolic solution of FDNB. The reaction mixture was kept in a 60°C water bath for 30 min with continuous stirring and then taken to dryness in a flash evaporator. After redissolution of the dry residue in 3 to 5 ml water and exhaustive extraction with ether the aqueous phase again was dried under reduced pressure and the DNP-murein was hydrolyzed as given below.

For reduction with sodium borohydride of terminal amino sugar residues in murein and degradation products of murein we used

in essential the procedure of Leyh-Bouille et al. (6) and Tipper et al. (7).
Amino sugars and amino acids were quantitated on an automatic amino acid analyzer BC 200 (BioCal Instrument G.m.b.H., München-Gräfelfing, Germany). All samples were hydrolyzed in 4 N HCl at 105°C for 14 hrs.

RESULTS AND DISCUSSION:

Quantitative determinations of peptide crosslinkage were made by reacting isolated mureins with FDNB and assaying the dinitrophenylated product for the amount of residual Dap which had not been converted to mono-DNP-Dap because it had been involved in crosslink formation. Mureins of the following organisms were studied: rod-shaped Proteus mirabilis strains VI, 19, and 1 Ca/5, and unstable L-form strains 1 Ca/pen and 19/5, grown in the presence of penicillin. The partners of two pairs of bacterial and L-form strains were closely related to each other but the duration of maintenance of the L-form strains on penicillin medium was very different. L-strain 1 Ca/pen was isolated in 1955 by O. and G. Kandler from P.mirabilis D 52 and has since been subcultured on agar medium containing 200 units penicillin G per ml. The rod-shaped strain 1 Ca/5 was derived from 1 Ca/pen by reversal on penicillin free medium. L-strain 19/5 was obtained in 1968 in our laboratory from P.mirabilis strain 19, by L-conversion on penicillin agar (8). Table 1 shows the molar ratio of amino acids in the different mureins before and after treatment with DNFB.
Dap was the only amino acid reacting with DNFB in all samples. The amount of non-dinitrophenylated Dap engaged in peptide crosslinkage was identical in mureins from rod-shaped strains and mureins synthesized in the presence of penicillin. There is no evidence of reduced peptide crosslinkage resulting from the presence of penicillin in the medium. (A slight deviation from the normal molar ratio of Dap:Glu:Ala = 1:1:1.8 is due to the presence of contaminating protein in some of the murein samples.) In fact, the amount of crosslinkage in all normal and penicillin damaged Proteus murein coincides with the value determined for the murein of E.coli by Takebe (9). Again, the analyses of L-form mureins give no indication of the alanine surplus which would

be expected from the postulated inhibition of transpeptidation by penicillin. Recently, Schwarz et al. (10) have reported that short-time action of penicillin on in vivo biosynthesis of murein in E.coli, strain W 173-25 does not induce a reduction of peptide crosslinkage or a retention of alanine in the polymer. Our data on the long term effect of penicillin on Proteus agree well with the results obtained in E.coli.

Table 1. Molar ratios of amino acids in Proteus murein before and after reaction with fluorodinitrobenzene.

	untreated murein			dinitrophenylated murein		
rod-shaped P.mirabilis	Glu	Ala	Dap	Glu	Ala	Dap
strain 19	1.11	1.95	1.00	1.11	1.98	0.32
strain VI	1.23	2.26	1.00	1.23	2.28	0.34
strain 1 Ca/5	1.08	1.92	1.00	1.08	1.80	0.34
L-form (+penicillin)						
strain 1 Ca/pen	1.05	1.98	1.00	1.05	1.88	0.32
strain 19/5	1.22	2.08	1.00	1.22	2.11	0.33

The amount of Dap : 1.00 in the untreated murein samples was used as reference throughout. The calculation of corresponding molar ratios for the dinitrophenylated murein was made by substituting as auxiliary references the values for Glu, as they were determined in untreated murein. Previous quantitative determinations had shown that the absolute amount of Glu in all mureins remained unchanged after dinitrophenylation.

Table 2. Molar ratios of amino sugars and amino acids in murein of P.mirabilis, strain VI, and in murein degraded with lysozyme and reduced with $NaBH_4$.

	Mur	GlcN	Glu	Ala	Dap
untreated murein	0.95	0.95	1.13	2.04	1.00
intact murein reduced with $NaBH_4$	0.90	0.93	1.11	2.01	1.00
lysozyme-degraded murein reduced with $NaBH_4$	0.58	0.94	1.13	2.00	1.00

A difference in the extent of peptide crosslinkage in normal and penicillin-damaged mureins of Proteus had first been suspected when it was found that the polymers are attacked in a different way by hen's egg-white lysozyme. This enzyme degrades the murein of the unstable L-form completely into the known muropeptide subunits and peptide-linked subunit dimers which were first identified by Primosigh et al. (11) and Pelzer (12) in lysozyme split products of E.coli B. In contrast, murein of rod-shaped P.mirabilis is broken down but not degraded to the same small fragments even by prolonged and repeated lysozyme treatment (3, 4). We have now obtained another explanation of this phenomenon.

One to 1.5 μmole of isolated murein of P.mirabilis strain VI was degraded by incubation with 200 μg hen's egg-white lysozyme (Sigma Chemical Comp., St.Louis, Mo.) in 2.0 ml 0.1 M ammonium acetate solution, pH 6.5 for 15 hrs. at 37°C. The extent of degradation of the glycan moiety of the murein was then determined by reducing the free end groups of N-acetylmuramic acid residues liberated by lysozyme action with $NaBH_4$, hydrolysing the reaction product and measuring the amount of residual non-reduced muramic acid on the automatic amino acid analyzer. The results are given in table 2.

Approximately 60 % of the muramic acid in the lysozyme split product remained unreduced. It is clear from these data that hen's egg-white lysozyme leaves a major portion of potentially lysozyme sensitive bonds in the glycan of normal Proteus murein undegraded. However, the same enzyme is able to effect an essentially complete breakdown of murein of the unstable L-form 1 Ca/pen and also of the murein of E.coli B to the disaccharide stage of the muropeptide subunits. A recently characterized N-acetylmuramidase from a Limax amoeba (Katz, Berger, and Martin, manuscript in preparation) surpasses hen's egg-white lysozyme in being able to degrade also the murein of P.mirabilis strain VI completely to the disaccharide stage. The structural features which make normal Proteus murein partially resistant to hen's egg-white lysozyme, in contrast to the murein of some strains of the unstable L-form, are presently under investigation.

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