THE AMINO ACID SEQUENCE OF THE L-GLUTAMIC ACID CONTAINING MUREINS OF MICROCOCCUS LUTEUS AND M.FREUDENREICHII

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The mureins (peptidoglycans) known so far, contain 1 mole of D-glutamic acid per mole of murein subunit (1, 2, 3). Studying the mureins of micrococci, we found two strains, which contain 2 moles of glutamic acid per subunit, one of the D- and one of the L-configuration. This paper describes experiments which show, that in both strains the D-glutamic acid is part of the tetrapeptide, attached to muramic acid as usually, while the L-glutamic acid takes part in the cross linkages. The interpeptide bridge between the carboxyl group of D-alanine of one tetrapeptide and the ε-amino group of lysine of another tetrapeptide consists of γ-L-glutamyl-glycine in M. luteus and of γ-L-glutamyl-L-alanine in M. freudenreichii.

Material and methods:

M. freudenreichii ATCC 407 and M. luteus ATCC 398 were grown in a yeast extract glucose broth (0,5 % yeast extract, 1 % peptone from casein, 0,5 % glucose, pH = 7.5) under aeration and harvested in the early stationary phase. Cell walls were prepared by the usual technique (4). For purification

the cell walls were incubated with trypsine for 24 hrs (CW-tryp). A part of the cell walls was extracted by TCA for 3 days at 4°C (CW-TCA) and an aliquot of this material was further extracted by hot formamide (CW-FA).

To separate amino acids, amino sugars and peptides, by paper chromatography, the following solvent systems were used:

I. Isopropanol - acetic acid - water = 75:1o:15
II. α-Picoline - conc. NH_4OH - water = 7o:2:28
III. n-Butanol - pyridine - acetic acid - water = 42o:28o:21:21o.

Quantitative amino acid analysis was performed by an amino acid analyser (Beckman). DNP-amino acids were identified by thin layer chromatography (cellulose powder) using the following solvent systems:

a) 1,5 M Phosphate buffer (pH = 6,o)
b) n-Propanol - o,2 % ammonia = 8:2.

The configuration of the amino acids was determined by measuring the ORD of the DNP derivatives (5). The quantitative determination of the L- and D-isomeres of alanine and of L-glutamic acid was carried out enzymatically (6). D-glutamic acid was measured by determining the difference between the total amount (measured as DNP-derivative) and L-glutamic acid.

Peptides from the acid partial hydrolysates were isolated by repeated one-dimensional paper chromatography in solvent systems I, II, III and identified by determining the amino acid composition and the N-terminal amino acid as described recently (4, 7). The tripeptides in addition, were subjected to partial hydrolysis and the arising dipeptides were identified.

Results:

A) The amino acid content and the N- and C-terminal amino acids. As shown in Tab. 1 the overall amino acid composition of the cell walls of both organisms differs from all other organisms investigated so far (1, 2) by the high content of glutamic acid which amounts to about two mole per mole of lysine. Both organisms differ from each other in respect to the alanine content and the occurrence of glycine. The ratio alanine:lysine is about 3,o in M. freudenreichii, but 2,o in M. luteus.

Tab.1: The absolut amount and the molar ratios of amino acids and amino sugars of cell walls from both micrococci.

CW-preparation		mM/g cell wall						Molar ratio Lys = 1,o		
		Lys	Glu	Ala	Gly	Mur	$GlcNH_2$	Glu	Ala	Gly
M.freudenreichii	TRYP	o,44	o,82	1,15	-	o,38	o,36	1,86	2,6	-
	TCA	o,53	o,93	1,35	-	o,41	o,38	1,76	2,56	-
M. luteus	TRYP	o,32	o,69	o,63	o,31	o,26	o,25	2,14	1,97	o,97
	TCA	o,39	o,85	o,81	o,41	o,29	o,26	2,16	2,o6	1,o5

While M. luteus contains 1 mole of glycine per subunit, the murein of M. freudenreichii is free of glycine.

Both organisms contain more than 1 mole but less than 2 moles of ammonia per mole of lysine, indicating that one of the two glutamic acids is amidated as known from other organisms (2).

The hydrolysis of dinitrophenylated cell walls (CW-tryp and CW-TCA) yielded DNP-glutamic acid as the only DNP amino acid. The photometric determination of DNP-glutamic acid showed,

that in M. luteus 1o % and in M. freudenreichii 7 % of the total glutamic acid is N-terminal.

The hydrazinolysis of the cell walls (CW-tryp) yielded free lysine and free alanine in both organisms. In M. luteus 3 % of alanine and 7 % of lysine and in M. freudenreichii 1 % of alanine and 8 % of lysine are C-terminal. These are minimum amounts since part of the lysine is destroyed during hydrazinolysis (8).

The quantitative determination of L- and D-alanine showed a ratio of 1 D- to 1,o5 L-alanine in M. luteus and 1 D- to 2,25 L-alanine in M. freudenreichii. The configuration of lysine was found to be L- while the glutamic acid, isolated from the total hydrolysate was inactive. In contrast, the DNP-glutamic acid derived from the hydrolysate of the dinitrophenylated cell walls proved to be L-glutamic acid.

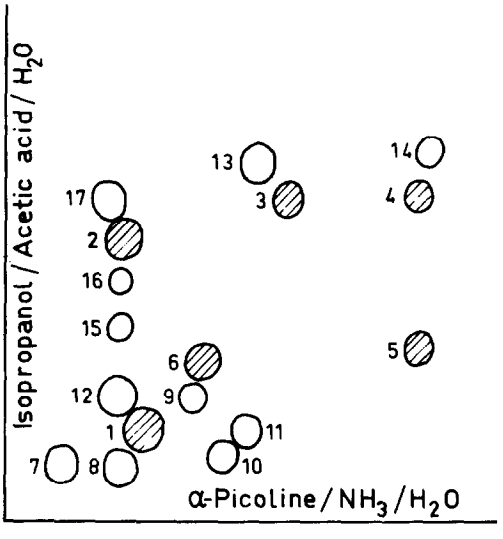

Fig.1: Paper chromatogram of an acid partial hydrolysate of the cell wall of M. luteus. 1 = L-lysine, 2 = glutamic acid, 3 = alanine, 4 = muramic acid, 5 = glucosamine, 6 = glycine, 7 = γ-D-Glu-L-Lys, 8 = L-Ala-γ-D-Glu-L-Lys, 9 = L-Lys-D-Ala, 1o = N^{ε}-Gly-L-Lys, 11 = N^{ε}-Gly-L-Lys-D-Ala, 12 = L-Lys-D-Ala-L-Glu, 13 = Mur-L-Ala-D-Glu, 14 = Mur-L-Ala, 15 = γ-L-Glu-Gly, 16 = D-Ala-γ-L-Glu-Gly, 17 = L-Ala-D-Glu and D-Ala-L-Glu (Nr. 13,14,15 and 16 are found after short hydrolysis of 1o min only).

B) The amino acid sequence. To elucidate the amino acid sequence both cell walls (CW-TCA) were partially hydrolysed (4 N HCl, 100°C, 10-60 min). Fig. 1 and 2 show the two-dimentional paper chromatograms and the various peptides identified.

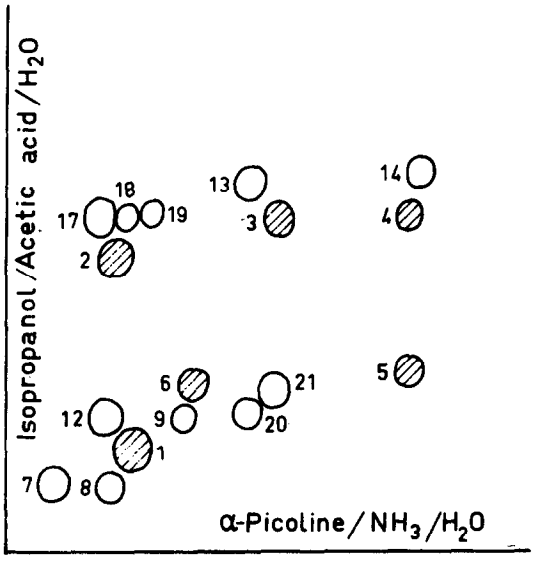

Fig.2: Like Fig.1 but for M. freudenreichii. 1-17 see Fig.1. 18 = γ-L-Glu-L-Ala, 19 = D-Ala-γ-L-Glu-L-Ala, 20 = N^{ε}-L-Ala-L-Lys, 21 $^{\varepsilon}$= N -L-Ala-L-Lys-D-Ala (Nr. 18 and 19 are found after short hydrolysis of 10 min only).

The occurrence of peptide Nr. 14, 13, 17, 7, 8, 9 and of one mole ammonia per mole lysine indicate that both mureins contain the usual subunit muramyl-L-alanyl-γ-D-isoglutamyl-L-lysyl-D-alanine.

To prove that the peptide Glu-Lys contains the γ-bond, this peptide was split by hydrazinolysis and the γ-hydrazide of glutamic acid was determined by paper chromatography in solvent system III. In addition the photolysis of the DNP-peptide in UV light was measured by following the absorption increase at 284 nm (9).

Since the total hydrolysate of the cell walls contained both isomeres of glutamic acid and alanine, we determined the amount of these isomeres in the peptide Ala-Glu enzymatically. It was found, that about 2/3 of alanine was of the L-, the rest of the D-configuration. In case of glutamic acid the ratio was reverse. This indicates that the peptides L-Ala-D-Glu and D-Ala-L-Glu are present at a ratio 2:1.

The occurrence of D-Ala-L-Glu indicates that L-glutamic acid is involved in the cross-linkage in both organisms. The two mureins differ from each other with respect to the second amino acid of the interpeptide bridge. In case of M. luteus, this amino acid is glycine. It is bound to the γ-carboxyl group of L-glutamic acid as shown by the occurrence of the peptide γ-L-Glu-Gly. Again, the γ-bond was demonstrated by the photolysis of the DNP-derivative as well as by the chromatographic identification of the γ-hydrazide of glutamic acid in the hydrazinolysate. The peptides N^{ε}-Gly-L-Lys and N^{ε}-Gly-L-Lys-D-Ala further demonstrate, that glycine is bound to the -amino group of lysine. A typical fragment of the murein of M. luteus is shown

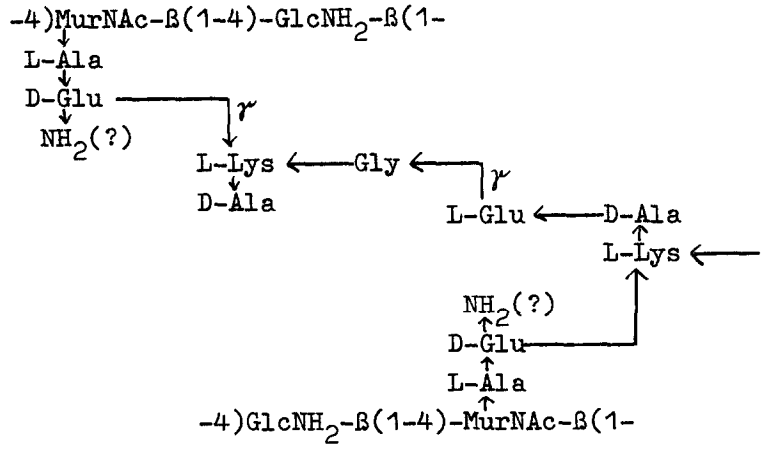

Fig.3: Fragment of the murein of M. luteus.

in Fig. 3. In analogy with other mureins it is assumed, that the D-glutamic acid of the tetrapeptide but not L-glutamic acid of the interpeptide bridge is amidated. However, there is no direct evidence for this.

In the whole cell wall, about 2o % of the L-glutamic acid is not crosslinked as indicated by the fact, that lo % of the total glutamic acid is N-terminal. In addition, some of the tetrapeptides are incomplete since 7 % of L-lysine is C-terminal.

In the case of M. freudenreichii the position of glycine is taken by L-alanine. This is demonstrated most clearly by the occurrence of the peptides D-Ala-L-Glu, γ-L-Glu-L-Ala, D-Ala-γ-L-Glu-L-Ala, N^{ε}-L-Ala-L-Lys, N^{ε}-L-Ala-L-Lys-D-Ala and L-Lys-D-Ala-L-Glu. The amino acid sequence of the murein of M.freudenreichii can be written as shown in Fig. 4. The murein of M. freudenreichii shows about the same degree of incompleteness as that of M. luteus since similar amounts of N-terminal L-glutamic acid and C-terminal D-alanine and L-lysine were found.

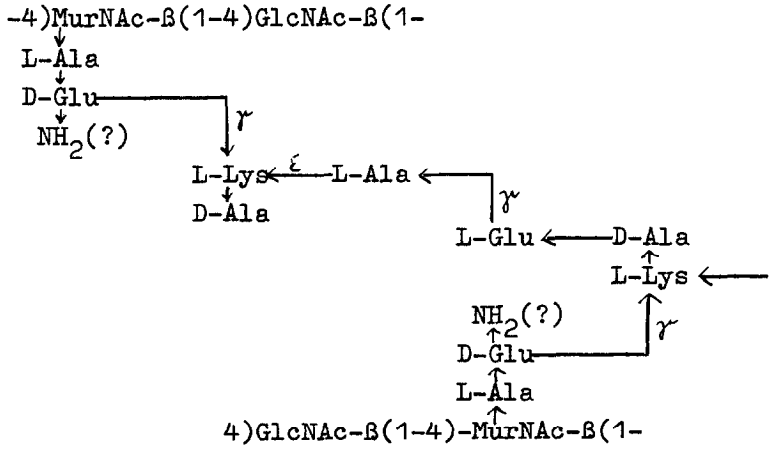

Fig.4: Fragment of the murein of M. freudenreichii.

Discussion:

So far, D-aspartic acid was the only dicarboxylic amino acid, known to be involved in the crosslinkage of mureins especially in lactobacilli and streptococci (11, 12, 13). While in these organisms D-aspartic acid is the only amino acid in the interpeptide bridge and therefore bound directly to the ε-amino group of lysine, in the mureins described in this paper, alanine or glycine is inserted between the ε-amino group of lysine and the γ-carboxyl group of L-glutamic acid.

The common feature of both, the D-aspartic acid and L-glutamic acid containing mureins, is the unusual γ- or ß-bond of glutamic and aspartic acid, respectively. A murein very similar to that of M. luteus was found in a strain of Sarcina ureae in our laboratory (unpublished results). It shows the same amino acid sequence, but both glutamic acids are of the D-configuration.

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