Glycine Amide a Constituent of the Murein of *Arthrobacter atrocyaneus*

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The amino acid sequence of the murein of *A. atrocyaneus* was determined. The peptide subunits L-Ala- γ -D-Glu-L-Lys-D-Ala are crosslinked by the interpeptide bridge L-Ala-L-Ala-L-Ser. The α -carboxyl group of D-glutamic acid is substituted by glycine amide which was never found in any other murein so far.

Amide groups are very common in the mureins (peptidoglycans) described so far. The amides most frequently found are isoglutamine and isoasparagine¹. In *Lactobacillus plantarum*^{2, 3} and in *Corynebacterium diphtheriae*⁴ the carboxyl group of diaminopimelic acid which is not involved in the peptide bond of the peptide subunit is also amidated.

The present paper describes the occurrence of glycine amide bound to the α -carboxyl group of glutamic acid.

1. Methods

A. atrocyaneus ATCC 13752 was grown in yeast extract-dextrose-broth (1% peptone from casein, 0.5% yeast extract (Cenovis), 0.5% glucose, pH = 7.2) at 30 °C and harvested in the stationary phase. Prior to harvesting the cell suspension was kept at 100 °C for 30 min to inactivate autolytic enzymes. Cell walls were prepared by the usual technique⁵. Paper chromatography was carried out on Schleicher and Schüll 2043b paper in the following solvent systems (v/v):

- I. Isopropanol-acetic-acid-water = 75:10:15.
- II. α -Picoline-ammonia solution 25%-water = 70: 2:28.

Quantitative amino acid analysis was carried out by an amino acid analyzer.

The determination of endgroups of the murein and the peptides which were isolated from acid partial hydrolysates by paper chromatography were performed as described recently⁵.

The configuration of glutamic acid and lysine, was determined by measuring the ORD of the DNP derivatives at the shoulder of the absorption spectrum at 418 nm. The configuration of serine was determined by taking the difference between the total amount of serine and the amount of D-serine which was measured enzymatically by using D-amino acid oxydase. The

quantitative determination of L- and D-alanine was carried out by enzymatic methods⁶.

DNP-amino acids were separated by thin layer chromatography in the following solvent systems (v/v):

- a) chloroform-methanol-acetic acid = 95:5:1,
- b) propanol-ammonia solution 0.2% = 8:2
- c) 1.5 molar phosphate buffer pH 6.0.

The cell walls were extracted by trichloroacetic acid for 3 days at 4 °C to remove teichoic acid and by formamide at 170 °C for 20 min to remove polysaccharides.

Determination of phosphate was carried out according to the method of FISKE and SUBBAROW⁷.

2. Results

2.1. The main components of the cell wall

The phosphate content of the cell walls (CW-TRYP) was only 0.5 per cent. There was no decrease after extraction by trichloroacetic acid. Thus, the cell walls of *A. atrocyaneus* contain no teichoic acid. The murein content of the cell walls after tryptic digestion was only 25 per cent. This number was calculated from the amino acid content of the preparation. After extraction by trichloroacetic acid the murein content increased to 42 per cent. A further increase to 72% was found after extraction by hot formamide (Table 1).

Polysaccharides could be precipitated in both extracts. Paper chromatography of the acid hydrolysate of the extracts demonstrated the presence of glucose, galactose, mannose and glucosamine. KEDDIE et al.⁸ found the same sugars in his strain of *A. atrocyaneus* with the exception of mannose.

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³ T. MATSUDA, S. KOTTANI, and K. KATO, Biochem. J. **11**, 111 [1968].

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⁵ K. H. SCHLEIFER and O. KANDLER, Arch. Mikrobiol. **57**, 335 [1967].

⁶ H. U. BERGMAYER, Methoden der enzymatischen Analyse, Verlag Chemie, Weinheim 1962.

⁷ C. H. FISKE and G. SUBBAROW, J. biol. Chemistry **66**, 375 [1925].

⁸ R. M. KEDDIE, B. G. S. LEASK, and J. M. GRAINGER, J. appl. Bacteriol. **29**, 17 [1966].

	<i>n</i>	[%]	Glu	Ala	Gly	Ser	Lys	Orn	Mur	GlcNH ₂	NH ₃
CW-TRYP	5	25	1.0	3.9	1.1	0.95	0.95	0.10	0.8	2.9	1.35
CW-TCA	1	42	1.0	3.84	1.06	0.95	0.94	0	0.8	2.46	1.25
CW-FA	1	73	1.0	3.75	1.0	0.85	0.93	0	0.87	1.48	1.4

Table 1. Molar ratio of the amino acids and amino sugars in the total hydrolysate of cell. CW-TRYP = cell walls purified by tryptic digestion. CW-TCA = trichloroacetic acid extracted cell walls. CW-FA = hot formamide extracted cell walls. *n* = numbers of the preparations. O = not determined.

The main components of the cell wall preparation (CW-TRYP) are obviously polysaccharides (about 75%) and murein (about 25%).

2.2. The amino acid composition of the murein

The amino acid composition of the cell wall is given in table 1. Mur, GlcNH₂, Lys + Orn, Glu, Gly, Ser, Ala, were present in a molar ratio of about 1 : 2.5 : 1 : 1 : 1 : 1 : 4. The portion of ornithine was about 10% of the total diamino acid.

Glutamic acid was present in the D-form, while serine, and lysine were in the L-form. Alanine was found as both isomeres showing a quantitative ratio D-Ala : L-Ala of 1 : 3.96 (average of 3 determinations).

Little more than one mole of ammonia was found per mole of glutamic acid. When the cell walls were hydrolysed for only 4 hours almost exactly one mole of ammonia was liberated. This is to be expected when ammonia is derived from an amide. The slow increase of ammonia during prolonged hydrolysis is probably caused by the breakdown of amino sugars.

Glucosamine is present in an excess compared to muramic acid in the CW-TRYP preparation. The amount drops after TCA extraction. Further extraction with formamide removes about half of the glucosamine, thus indicating that the excess is not part of the murein, but of the polysaccharide.

2.3. The N- and C-terminal amino acids

In the hydrolysate of DNP cell walls the DNP-amino acids were determined by two methods. 1. Photometric determination after isolation by thin layer chromatography, 2. comparison of the amino acid content of the hydrolysate of dinitrophenylated and not dinitrophenylated cell walls. Both methods gave similar results (Table 2). The values obtained

by method 2 of course are much more uncertain than that of method 1. The main N-terminal amino acid is L-alanine, followed by L-serine and L-lysine (ϵ -amino group).

	moles/mole Glu		C-terminal
	N-terminal Meth. 1	Meth. 2	
Ala	0.25	0.34	0.2
Lys	0.06	0.10	0.09
Ser	0.07	0.04	0.0
Gly	0.0	0.0	0.04

Table 2. N- and C-terminal groups.

Cell walls (CW-TRYP) were subjected to hydrazinolysis with water-free hydrazine for 20 h at 80 °C. After extraction by benzaldehyde the residual traces of hydrazides were removed from the hydrazinolysate by ion exchange column chromatography. The free amino acids in the effluent were determined by the amino acid analyzer. Besides alanine, lysine and a very small amount of glycine, an unknown peak close to lysine was found. This substance could be identified as the dipeptide ϵ -L-Ser-L-Lys. According to the structure of the murein given in Fig. 2 this peptide may arise when the C-terminal D-alanine of a peptide subunit is split off and L-lysine becomes the C-terminus. The amount of this peptide was added to the amount of C-terminal lysine. The data are given in table 2. It is well known, that hydrazinolysis leads to partial destruction of amino acids⁹. This destruction rate was determined by the hydrazinolysis of a cell wall of known composition (*Micrococcus lysodeikticus*) which contains C-terminal glycine, bound to the α -carboxyl group of glutamic acid. 80% of the total glycine was found after hydrazinolysis which agrees with the result of TIPPER et al.¹⁰. The data in table 2 are therefore corrected for a destruction factor of 20 per cent.

⁹ V. BRAUN and W. A. SCHRÖDER, Arch. Biochem. Biophysics 118, 241 [1967].

¹⁰ D. J. TIPPER, W. KATZ, J. L. STROMINGER, and J. M. GHUYSEN, Biochemistry 6, 911 [1967].

2.4. The amino acid sequence of the murein

A typical two dimensional paper chromatogram of an acid partial hydrolysate of the murein is shown in Fig. 1. The peptides Mur-L-Ala, Mur-L-Ala-D-Glu, γ -D-Glu-L-Lys and L-Lys-D-Ala indicate that the peptide subunit is muramyl-L-alanyl- γ -D-glutamyl-L-lysyl-D-alanine. Glycine is bound to the α -carboxyl group of D-glutamic acid, as demonstrated by the appearance of the dipeptide D-Glu-Gly.

Fig. 1. Scheme of a paper chromatogram of an acid partial hydrolysate (4 N HCl, 100 °C, 45 min.) of cell walls of *A. atrocyaneus*. 1 = L-Lys, 2 = D-Glu, 3 = Gly, 4 = L-Ser, 5 = DL-Ala, 6 = Mur, 7 = GlcNH₂, 8 = α -D-Glu-Gly, 9 = L-Ala-D-Glu, 10 = D-Ala-L-Ala, 11 = L-Ala-L-Ala, 12 = Mur-L-Ala, 13 = pink artefact of 8, 14 = γ -D-Glu-Lys, 15 = L-Lys-D-Ala, 16 = ϵ -L-Ser-L-Lys, 17 = ϵ -L-Ser-L-Lys-D-Ala, 18 = Mur-L-Ala-D-Glu, 19 = L-Ala-L-Ala-L-Ala (Nos. 12 and 18 appear in 20 min. hydrolysates only).

The mode of crosslinkage can be deduced from the existence of the peptides ϵ -L-Ser-L-Lys and ϵ -L-Ser-L-Lys-D-Ala on the one hand and D-Ala-L-Ala and L-Ala-L-Ala on the other hand. The interpeptide bridge resembles that in *Lactobacillus viridescens* ssp. minor¹¹.

The structure of the murein of *A. atrocyaneus* can be written as shown in Fig. 2. The interpeptide bridge L-alanyl-L-alanyl-L-serine is bound by its amino group to the C-terminal D-alanine of a peptide subunit and with its carboxyl group to the ϵ -amino group of L-lysine of an adjacent peptide subunit. The dipeptide L-Ala-L-Ser which should also occur in the partial hydrolysate was not iso-

lated, however, it was found in the case of *L. viridescens*¹¹. This dipeptide occurs in very small amounts, since the bond between L-serine and the ϵ -amino group of L-lysine is very stable. Correspondingly, the probability for the occurrence of L-Ala-L-Ser is much less than that for the formation of ϵ -L-Ser-L-Lys and L-Ala-L-Ala.

As mentioned in chapter 2.2, the murein contains 1 mole of ammonia which is split off already after short hydrolysis. The only possible position of this ammonia is the carboxyl group of glycine. In contrast to the murein of *M. lysodeikticus* which contains glycine at the same position the hydrazinolysis of the murein of *A. atrocyaneus* yielded almost no free glycine. This is in agreement with the assumption that glycine is amidated. Glycine amide is only rarely found in nature i. e. in the hormones Vasopressin and Oxytocin.

The scheme of the murein structure presented in Fig. 2 is not in full agreement with the data in table 1 and 2. The genuine murein differs from the scheme as follows:

Fig. 2. Scheme of the amino acid sequence of the murein of *A. atrocyaneus*.

1. Occurrence of ornithine: Since ornithine is not a component of protein, the small amount found in the hydrolysate of the cell wall is not an impurity. The peptides ϵ -L-Ser-L-Lys, ϵ -L-Ser-L-Lys-D-Ala and L-Lys-D-Ala isolated by paper chromatography also contained small amounts of ornithine and the sum of lysine and ornithine was equal to the amount of

¹¹ O. KANDLER, R. PLAPP, and W. HOLZAPFEL, Biochim. biophysica Acta [Amsterdam] **147**, 252 [1967].

serine or of alanine. This demonstrates that about 10% of the lysine of the peptide subunit is replaced by ornithine. Such a mixture of lysine or ornithine containing peptide subunits was also recently found in the murein of some bifidobacteria¹².

2. The occurrence of 0.25 mole of N-terminal L-alanine per mole of glutamic acid shows that about 25% of the interpeptide bridge are not crosslinked. Another part of the interpeptide bridges is obviously incomplete, since about 0.05 mole of L-serine are N-terminal. In addition 6–10% of the ϵ -amino groups of L-lysine are not substituted. These data mean that only 60% of the peptide subunits are crosslinked.

3. In agreement with the uncomplete crosslinking, some D-alanine of the peptides subunits is C-terminal as shown by the result of the hydrazinolysis (table 2). Since the cell wall precursor (UDP-muramyl-pentapeptide) contains a di-D-alanine, it is to be expected that the uncrosslinked peptide subunits contain a C-terminal di-D-alanine. This is probably not the case, since the ratio D-Ala : L-Ala in the total hydrolysate is 1 : 4, instead of the theoretical value 1 : 3. When the second mole of D-alanine would partially remain with the peptide subunits, the relative portion of L-alanine would be decreased below 3.0, and not increased. The loss of the second molecule of D-alanine from the uncrosslinked peptide subunits is very common also in other mureins¹. It is probably caused by the action of a carboxypeptidase which may split off even both molecules of D-alanine, therefore leading to a peptide subunit with a C-terminal L-lysine. The latter case is also known in *A. atrocyaneus*, shown by the occurrence of about 10% C-terminal lysine.

The absence of about 10–15% of L-alanine of the interpeptide bridge as well as the loss of 20% of the D-alanine of the peptide subunits leads to a molar ratio alanine : D-glutamic acid of less than 4.0, namely 3.6 and to a ratio D-Ala : L-Ala of 3.5. The actual numbers are 3.9 and 3.96 respectively. This would best be explained if some of the interpeptide bridges contain three instead of two molecules of L-alanine. In fact small amounts of a tri-L-alanine were found in the partial hydrolysate (Fig. 1). It was identified by cochromatography with authentic tri-L-alanine on paper and on the column.

If these various "failures" of the murein structure are taken into account, the data in Tab. 1 and 2 are in full agreement with the scheme given in Fig. 2.

3. Discussion

The amino acid sequence of the murein of *A. atrocyaneus* is unique within the coryneform bacteria¹³. The interpeptide bridge L-alanyl-L-alanyl-L-serine has only been found in *Lactobacillus viridescens* ssp. minor, some bifidobacteria, and in many species of *Leuconostoc*¹⁴. In all these cases, however, the α -carboxyl group of D-glutamic acid is amidated, not substituted by glycine amide. A similar substitution of glutamic acid was found recently in another coryneform bacteria. The murein of *A. spec.* NCIB 9423 was shown to contain alanine amide instead of glycine amide¹³.

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¹² W. H. HOLZAPFEL and O. KANDLER, Z. Naturforsch. **24 b**, 1524 [1969].

¹³ F. FIEDLER, K. H. SCHLEIFER, B. CZIHARZ, E. INTERSCHICK, and O. KANDLER, Folia microbiol. [Praha], in press.

¹⁴ O. KINDLER, Int. J. syst. Bacteriol., in press.