ISOLATION AND MASS SPECTROMETRIC IDENTIFICATION OF THE PEPTIDE SUBUNITS OF MYCOBACTERIAL CELL WALLS[§].

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SUMMARY. The same diamidated tetrapeptide L-Ala$^{\gamma}$-Gln-meso-DAP-(NH_2)-D-Ala has been isolated from the lysate by Myxobacter AL_1 enzyme of the peptidoglycan of Mycobacterium smegmatis, M. phlei, M. tuberculosis BCG and Corynebacterium fermentans. Its structure has been established by mass spectrometry after N-acetylation and permethylation (see formula 1) and by chemical methods.

Mild acid hydrolysis of delipidated cell walls of Mycobacteria breaks the linkage between the arabinogalactan-mycolic acid polymer and the peptidoglycan (1,2,3,4) which can then be solubilized by Myxobacter AL_1 enzyme (1,4,5) ; the fragments resulting from the action of this enzyme have been separated according to their molecular weight by filtration through Sephadex G 50 and/or G 25 (1,4).

The last fraction of these Sephadex columns contains monomers of the peptide moiety of the peptidoglycan : their purification and the establishment of their structure are reported in the present paper. Cell walls of M. smegmatis, M. phlei, M. tuberculosis BCG and C. fermentans have been studied : this latter organism was investigated to make a link with the work of Kato et al. (6) on cell walls of Corynebacteria.

METHODS. Delipidated cell walls were prepared as described in (1) and (4). Mild acid hydrolysis of delipidated cell walls was performed with 0.1 N HCl, for 12 h at 60° (4). Incubation with Myxobacter AL_1 enzyme was performed as described in (4), high voltage electrophoresis of peptides as described in (7).

Amino acid composition of the peptides was determined after total acid hydrolysis (HCl 6 N, 15 h, 110°) either with a Technicon or with a Beckman amino acid analyser.

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Amide ammonia was measured after total hydrolysis, using a Beckman amino acid analyser equipped with a filter for ammonia ; adequate blanks were done by evaporating an amount of HCl equal to that used for the hydrolysis, then adding the same aliquot of the peptide solution as used for the analysis and determining the ammonia content of the blank.

The peptides were purified on a sulfonic acid cation exchange resin (Beckman PA 35) (8). The sample, concentrated to 2 ml under reduced pressure, was adjusted to pH 2.5 with formic acid and applied to a column (ϕ = 1 cm, h = 7 cm) of the resin equilibrated with a pyridine-acetic acid buffer pH 3.1, 0.2 M in pyridine ; 40 ml of this buffer were used as first eluent and then a linear gradient was applied beginning with this buffer (300 ml) in the mixing vessel and a pyridine-acetic acid buffer pH 5.1, 2 M in pyridine, in the reservoir. The column was kept at 50° and fractions of 2.5 ml were collected at a flow rate of 10 ml/h ; 0.025 ml aliquots of each other tube were hydrolyzed by 0.2 ml of 2.5 N NaOH at 100° for 150 min. ; the solutions were neutralized with 0.2 ml of 30 % acetic acid and a ninhydrin reaction was performed.

The configuration of Ala was determined as described in (9). The configuration of the N-terminal Ala in the diamidated peptides of M. smegmatis was proved to be L- by showing that the Ala-Gln linkage is hydrolysed by beef eye lens amino-peptidase. The configuration of DAP was determined as described in (10) and was found to be meso.

The peptides were desalted by filtration in 0.1 N acetic acid through a column (ϕ = 1.5 cm ; h = 85 cm) of Sephadex G 15.

Deamidation of the peptides was performed by incubating the peptide in 10 N HCl for 6 h at 25° (8), then separating the deamidated peptides from the products of their partial hydrolysis by high voltage electrophoresis at pH 4.0.

Mass spectrometry was performed with a AEI, Model MS 9 mass spectrometer after N-acetylation and permethylation according to Hakomori's method (11, 12), on 0.5 to 1 μmole of peptide.

RESULTS.

The soluble peptidoglycan fragments resulting from the action of Myxobacter AL_1 enzyme on cell walls of M. phlei, M. tuberculosis BCG and C. fermentans were filtered on Sephadex G 25 : the elution patterns of the Sephadex columns have already been published (fig. 1, ref. 4). The peptidoglycan fragments of M. smegmatis were filtered on two connected columns of Sephadex G 50 and G 25, giving an elution pattern similar to Fig. 1, ref. 1.

The last peak of each of these Sephadex filtrates contains the monomers of the peptide moiety of the peptidoglycan. An aliquot of each of these peaks was chromatographed on the Beckman PA 35 column : the elution pattern obtained for M. smegmatis is given in Fig. 1.

Peak 1 contains all the non peptidoglycan amino-acids of the sample in the form of peptides : it seems that these peptides were linked to the peptidoglycan in the cell wall and have been set free by the Myxobacter enzyme, the activity of which is not restricted to the peptidoglycan (13).

For peaks 3, 4 and 6, only the amino-acid composition after total acid hydrolysis was studied : they all contain Ala, Glu, DAP (DAP = 2,6-diaminopimelic acid) and Asp ; peak 3 contains also Ser and Gly, peak 4, Gly ; they might contain more than one peptide. Peak 7 was fractionated by chromatography on Whatman n° 1 paper in butanol-acetic acid-water (62-15-25) into two peptides

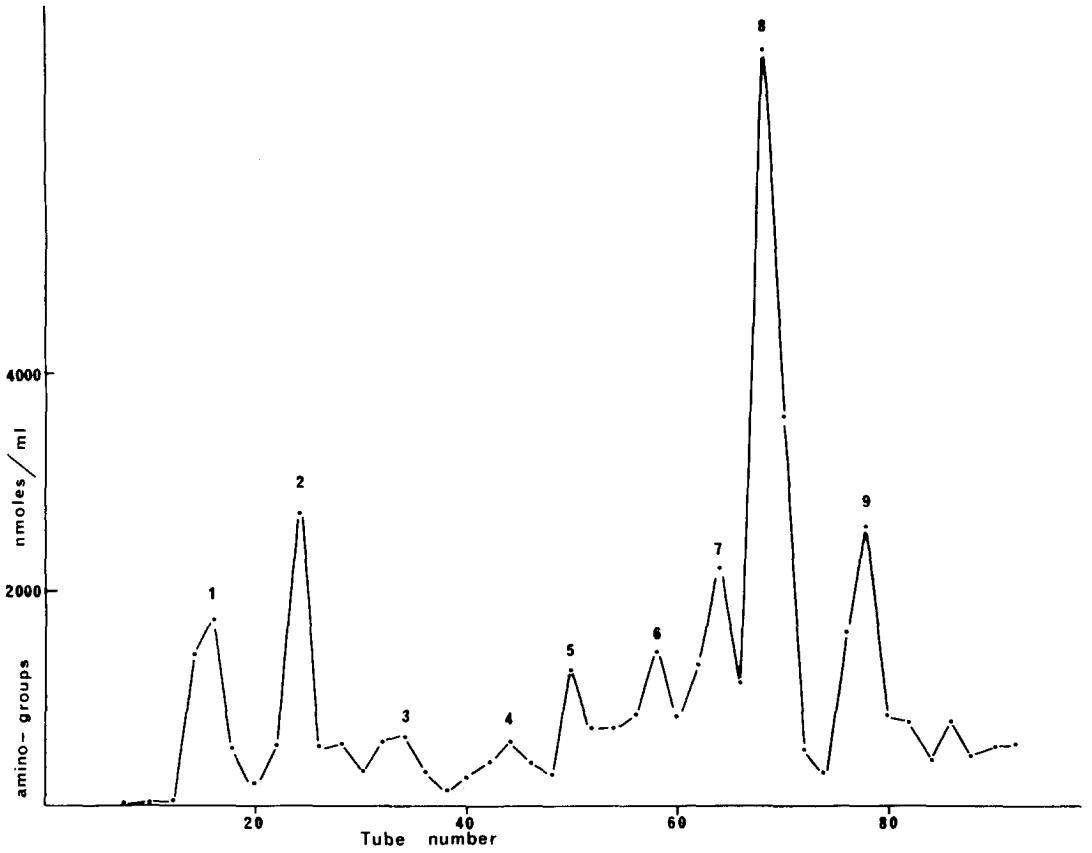

Figure 1 : Chromatography on a Beckman PA 35 column of the small peptides obtained by solubilization of M. smegmatis peptidoglycan with Myxobacter AL_1 enzyme.

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the faster one contains 2 Ala, 1 Glu and 1 DAP, the slower equimolar amounts of Ala, Glu, DAP and Asp ; their structure could not be studied due to their small amount.

Peaks 2, 5, 8 and 9 contain only one peptide each, as judged by thin layer chromatography on cellulose in butanol-acetic acid-water (62-15-25) and on silicagel in isobutyric acid-triethylamine-water (100-7-43) and high voltage electrophoresis at pH 1.9 and 4.0. As shown by amino-acid analysis, the peptide of peaks 2,5 and 8 are tetrapeptides Ala_2, Glu_1, DAP_1, and the peptide of peak 9 is a tripeptide Ala, Glu, DAP. Amide ammonia was determined on peptides 8 and 9 : they are diamidated.

For the other bacteria, only the major peaks of the Beckman PA 35 column were studied.

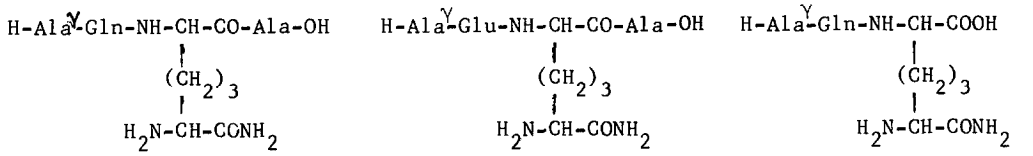
Two main peaks were eluted from the M. phlei column at the same position as peptides 8 and 9. They contain a single peptide having the same electropho-

retic mobility at pH 4.0 as peptides 8 and 9 and the same amino-acid composition. They will be called 8_p and 9_p.

One main peak was eluted from the M. tuberculosis BCG and C. fermentans columns at the same position as peak 8. For both bacteria, it contains a single peptide, similar to peptide 8. They will be called 8_t and 8_c.

All these peptides were desalted and analyzed by mass spectrometry.

Mass spectrometry - The mass spectra of the N-acetylated and permethylated derivatives of peptides 8, 8_p, 8_t and 8_c are similar and show molecular ion peaks at m/e 683 (see Fig. 2) and appropriate peaks due to peptide bond cleavage which show that they have structure 1*: the sequence peaks are observed at m/e = 567, 298 and 128 and allow the location of the amide groups. Of particular importance is the presence of an intense peak at m/e 484 (M-199) which can be interpreted as due to loss of the entire side-chain ($-(CH_2)_3-CH(MeNAc)-CONMe_2$) of DAP following cleavage a in 4 (Fig. 2), thereby confirming the linkage of Gln at position α to the carboxyl of DAP carrying the terminal Ala.



1 (peptide 8) 2 (peptide 5) 3 (peptide 9)

The N-acetylated and permethylated derivative of peptide 5 exhibits a molecular ion peak at m/e 670 and sequence peaks at m/e 554, 285 and 128. Moreover an intense peak at m/e 471 (M-199), due to a fragmentation similar to cleavage a in 4 established the exact location of the amide group in the DAP-residue. Peptide 5 thus has structure 2 ; it might be formed from peptide 8 by deamidation during the partial acid hydrolysis which is used to break the linkage between the arabinogalactan-mycolic acid complex and the peptidoglycan and which is necessary to make the peptidoglycan sensitive to the Myxobacter enzyme**.

* The presence of a γ-glutamyl linkage was determined by chemical analysis as described below.

** Peptide 2, which has not been analyzed by mass spectrometry, might be the other possible monoamide derived from peptide 8, the amide group being on Glu- ; this hypothesis is compatible with its behaviour on the Beckman PA 35 column and on electrophoresis at pH 4.0.

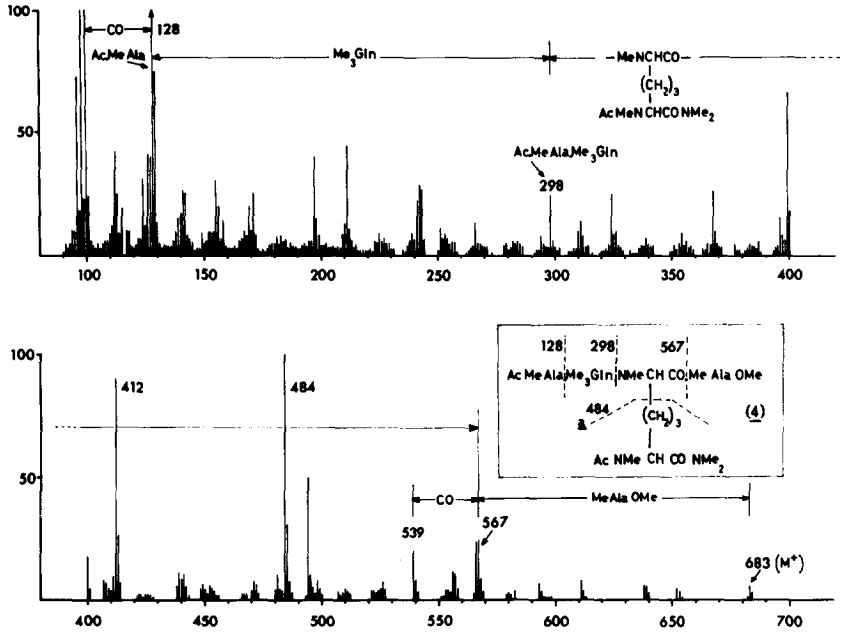


Figure 2 : Mass spectrum of the N-acetylated and permethylated tetrapeptide diamide of the cell wall peptidoglycan of M. smegmatis.

The derivatives of peptides 9 and 9_p show similar spectra with a molecular ion peak at m/e 598 and the sequence peaks at m/e 298 and 128. The peak due to loss of the DAP side-chain corresponding to cleavage a is now observed at m/e 399. Thus, peptide 9 and 9_p correspond to formula 3.

Beside the above-mentioned peaks, the mass spectra of these permethylated DAP-peptide derivatives show several other peaks of fairly high intensity. Further work is being continued to elaborate a detailed interpretation of these spectra.

The only structural detail not established by mass spectrometry is which of the carboxyl groups of glutamic acid is linked to DAP. It could however, be proved that it is the γ-carboxyl group in peptide 9 by comparing the electrophoretic mobility at pH 4.0 of the deamidated tripeptide corresponding to peptide 9 with the tripeptides L-Ala$^{\alpha}$-D-Glu-$^{(L)}$meso-DAP and L-Ala$^{\gamma}$-D-Glu-$^{(L)}$meso-DAP synthesized by P. Dezélée and E. Bricas (7,14). The deamidated peptide has the same electrophoretic mobility as the synthetic γ tripeptide.

Using the same procedure, tetrapeptide 8 was shown to have the same mobility at pH 4.0 after deamidation as the tetrapeptide L-Ala$^{\gamma}$-D-Glu-(L)-meso-DAP-(L)-D-Ala synthesized by P. Dezélée and E. Bricas (17) : the α isomer would not have had the same electrophoretic mobility.

For further comparison with the peptidoglycan of other bacteria, some stereochemical points were checked. As expected, the N-terminal Ala of peptides 8 and 9 is L-, the C-terminal Ala of peptide 8 is D-. DAP was shown to be meso in peptides 8, 8_p and 9.

Discussion.

Origin of the peptides studied. The peptides studied here were obtained as a result of the action of Myxobacter AL_1 enzyme on the peptidoglycan. It has not been proved unambiguously whether this enzyme is able to split interpeptide bridges (D-Ala- meso-DAP) in DAP containing peptidoglycans ; on such peptidoglycans, it acts mainly as an amidase (1,6) (splitting of muramic acid - L-Ala linkages). Thus the peptides studied here seem to be present in the cell wall as uncrosslinked monomers. According to Weidel and Pelzer (15) their origin can be ascribed to autolytic enzymes, supposed to be necessary for the regulation of the growth of the cell wall and present in all bacteria.

Structure of the peptides.

The structure of DAP peptides has been intensively studied in the last few years. In previous experiments, the identification of the carboxyl group of Glu linked to DAP was solved by Edman degradation (6) or hydrazinolysis (7,16) ; another approach, used here, is the comparison of the electrophoretic mobility of natural and synthetic peptides (14). The position of Glu and D-Ala on the DAP molecule has been solved indirectly by Edman degradation (7), by comparison of natural and synthetic peptides (17) and by other chemical methods (18).

The location of amide groups in peptidoglycan peptides has been done by Edman degradation (6,19) and dehydration-reduction (20,21).

Mass spectrometry offers a much easier approach to the last two of these structural problems. It allowed us to show that in Mycobacteria and Corynebacteria, Glu and D-Ala are linked to the same asymetric carbon of DAP and that in Mycobacteria, as in Corynebacteria (6), the amide groups of the diamidated tri- and tetrapeptides are located on Glu- and on the asymetric carbon of DAP not linked to Glu.

Thus, the peptide subunits of the peptidoglycan of Mycobacterial cell walls have the same structure as the DAP peptides of other species studied ; like those of Corynebacteria, they are diamidated.

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