STRUCTURAL STUDY OF THE POLY-L-GLUTAMIC ACID OF THE CELL WALL OF Mycobacterium tuberculosis var hominis, STRAIN BREVANNES.

Juana Wietzerbin[*], Florence Lederer[**] and Jean-François Petit[*].

[*]Institut de Biochimie, Université Paris-Sud, centre d'Orsay, France.
[**]Centre de Génétique Moléculaire du CNRS, 91-Gif-sur-Yvette, France.

Received November 7,1974

SUMMARY.

Trypsin-chymotrypsin-treated delipidated cell walls from M.tuberculosis Brévannes contain a poly-L-glutamic acid which can be extracted from the cell walls after partial acid hydrolysis as a molecule of about 35,000 daltons. Elementary analysis of this polymer suggests that it is at least partly amidated. The partial amidation and the α-linkage could be established up to the 14th residue by automatic Edman degradation.

INTRODUCTION.

Migliore et al. (1) have found that deproteinized and delipidated cell walls of human strains of M.tuberculosis contain large quantities of glutamic acid which Acharya (2) and Vilkas and Markovits (3) could extract as a polymer from M tuberculosis ,strain Brévannes and M.tuberculosis,strain Peurois respectively. We have recently shown (4) that trypsin-chymotrypsin-treated delipidated cell walls of BCG also contain non peptidoglycan glutamic acid which we obtained by partial acid hydrolysis, as L-glutamic acid oligopeptides where glutamic acid residues are linked by α-linkages.

We have postulated that they are part of a poly-α, L-glutamic acid which could play a role in the biological properties of BCG cell walls. Based on the abundance of non peptidoglycan glutamic acid, this polymer would constitute 2 % of the cell wall of BCG (4) and 8 % in our preparations of cell walls of M.tuberculosis Brévannes (a virulent strain).

To investigate further the structure of mycobacterial polyglutamic acid, we devised a method to obtain it from the cell walls of M.tuberculosis Brévannes under as mild conditions as possible. From data on the chemical composition we postulated that the polymer is partly amidated: this was confirmed by automatic Edman degradation.

MATERIALS AND METHODS.

Cell walls of M.tuberculosis strain Brévannes: cells grown on Sauton's medium in Roux bottles for 8 weeks at 37°, were kindly provided by Dr. J.Augier, Institut Pasteur (Paris). They were disrupted in an omnimixer (Virtis) for 30 minutes at 0° with glass beads. The suspension was centrifuged three times for 15 min. at 800 xg, to remove unbroken cells. The last supernatant was centrifuged for 50 min. at 27,000 xg, and the pellet containing the cell walls was suspended in 50 times its volume of 66 mM phosphate buffer pH 7.8 containing 0.05 % trypsin and 0.05 % chymotrypsin and incubated over-night. The cell walls were centrifuged and washed three times with 66 mM phosphate buffer and three times with water. The pellet was then delipidated with acetone (once), chloroform:methanol, 2:1 (twice) and dried.

Isolation of polyglutamic acid.

1) Partial acid hydrolysis of the cell walls: trypsin-chymotrypsin-treated delipidated cell walls, were suspended in 0.1 N HCl (10 mg/ml) and hydrolysed for 3 h at 110° in a sealed tube. The hydrolysate was then centrifuged; the supernatant was discarded and the pellet was delipidated overnight with chloroform/methanol 2:1.

2) Extraction of polyglutamic acid: the above described cell walls were suspended in 0.1 N NaOH and allowed to stand for 10 mn. at room temperature with stirring. Under these conditions the polyglutamic acid becomes soluble. The remaining cell walls were eliminated by centrifugation at 27,000 xg for 15 min. The alcaline solution was brought to pH 1 with HCl and allowed to stand at 4° for 30 min.: polyglutamic acid precipitates as a white powder which can be separated by centrifugation. It is slightly contaminated by peptidoglycan fragments. It was purified by redissolution in 0.1 N NaOH, 0.2 N NaCl, centrifugation, elimination of the pellet which contains most peptidoglycan contaminants and precipitation of the polyglutamic acid by acidification of the supernatant to pH 1 with HCl; this cycle was repeated three times, the last acid precipitate was washed once with 0.2 M NaCl, once with water and lyophilised.

Amino acid analyses:

Amino acids were measured after a 16 h hydrolysis at 110° in 6 N HCl,

using a Technicon amino acid analyser. The configuration of glutamic acid was determined as described in (4).

Determination of N-terminal amino acids: They were measured by the dansyl technique (5) .In a typical assay 700 µg of polyglutamic acid were dissolved in 300 µl of 0.4 M phosphate pH 8.5 containing 250 mg of urea and allowed to stand for 10 min. at room temperature; then were added 150 µl 0.4 M phosphate buffer pH 8.5, 250 µl dimethylformamide and 100 µl 0.2 M dansyl-chloride in acetonitrile. After 1 h at room temperature dansyl-polyglutamic acid was precipitated by addition of 5 ml 5 % trichloracetic acid.The precipitate was centrifuged, washed once with 0.1 N HCl and then hydrolysed in the presence of 600 µl 5.7 N HCl for 4 h. An aliquot was then withdrawn for amino acid analysis; the hydrolysate was dried under vacuum and the dansyl-amino acid dissolved in chloroform-methanol-acetic acid (7:2:2), spotted on silicagel plates (Schleicher and Schüll), chromatographed and the fluorescence measured as described in (5).

Sequence determination: The N-terminal sequence was determined by the automatic Edman degradation method (6) with a Socosi sequenator PS 100, using the normal protein program with two acid cleavage steps, and quadrol as the coupling buffer (6). The phenylthiohydantoins were identified by thin layer chromatography on silica gel with chloroform-methanol (9:1) and chloroform as solvents (7).

RESULTS.

Isolation of polyglutamic acid. As can be seen in Table I, trypsin-chymotrypsin-treated delipidated cell walls of M. tuberculosis Brévannes contain a ratio of Glu/DAP of 8.

Taking into account that in mycobacterial peptidoglycan the molar ratio of DAP/Glu/Ala is 1:1:1.5 (8) only 1/8 th of the Glu residues should belong to this polymer. We established by the enzymatic technique mentioned in Materials and Methods that this non peptidoglycan Glu has the L configuration. We tried to extract the polymer by various agents at room temperature: 0.5 N NaOH, 1 % NH_4OH, 1 % SDS and pepsin digestion (in 0.09 M sodium acetate pH 4.18, 0.18 M NaCl (9)), but without success. We thus postulated that this polymer was either covalently linked to the cell wall skeleton, or to lipids, polysaccharides or lipopolysaccharides which made it insoluble or that it

Table 1 : Amino acid composition of trypsin-chymotrypsin-treated delipidated cell walls of M.tuberculosis Brévannes.

Amino acids	nmoles/mg	molar ratio
Alanine	156	1.85
Glutamic acid	667	7.96
α, ε-diaminopimelic acid	84	1
Leucine	28	0.33
Glycine	35	0.42
Serine	31	0.37
Threonine	31	0.37
Aspartic acid	36	0.43
Valine	37	0.43

Table 2 : Amino acid analysis of three different samples of poly-L-glutamic acid of the cell walls of M.tuberculosis Brévannes.

Amino acids	Molar ratio relative to alanine		
	I	II	III
Glutamic acid	28	66.8	93.1
Alanine	1	1	1
Leucine	0.9	-	-
Glycine	0.6	-	0.9
Serine	0.4	0.9	-
Threonine	0.4	0.9	-
Aspartic acid	0.2	-	traces
α, ε-diaminopimelic acid	-	-	traces

Table 3 : Elementary analysis of two samples of poly-L-glutamic acid from the cell walls of M.tuberculosis Brévannes.

Found	C	H	N
Sample I	45.95	6.11	16.30
Sample II	46.07	5.96	15.10
Theoretical			
Free acid $(C_5H_7O_3N)_X$	46.66	5.42	10.80
Fully amidated $(C_5H_8O_2N_2)_X$	46.87	6.24	21.95

was too large a polymer to be soluble; we thus tried to extract it by as mild conditions as possible. We eventually succeeded using the following steps: 1) partial acid hydrolysis (0.1 N HCl, 3 h ,110°) of trypsin-chymotrypsin-treated delipidated cell walls, 2) delipidation by acetone and chloroform-methanol 2:1 , 3) alkaline extraction by 0.1 N NaOH. Under these conditions, most of the poly-L-glutamic acid of the cell walls becomes soluble. It is, however, slightly contaminated by peptidoglycan fragments. We could purify it by repeated precipitations at acid pH, followed by redissolution at basic pH as described in Methods.

Characterization. The yield is about 50 % of the theoretical value calculated from the L-Glu content of the cell walls. Its amino acid composition is given in Table 2. More than 95 % of the amino acids are Glu, only one α-amino-group per about 270 Glu residues reacts with dansylchloride. No other N-terminal group can be detected. From these data one can assume that the average molecular weight of the polymer is about 35,000 daltons.

As synthetic poly-α, L-glutamic acid, dissolved in 90 mM sodium acetate pH 4.18, 0.18 M NaCl, is known to be hydrolysed by pepsin into oligopeptides (9), we incubated the synthetic and the isolated polymer with this enzyme. Hydrolysis of the synthetic polymer gave rise, as expected, to oligopeptides separable by paper electrophoresis but the natural polymer was apparently not hydrolysed. We thus submitted the bacterial polymer to elementary analysis (Table 3). The results, compared with the theoretical values for polyglutamic acid, show that there is an excess of nitrogen which can be explained by a partial amidation of the polymer; this could be the reason of its insensitivity to pepsin.

Edman degradation. A first run in the sequenator with about 5 mg (0.16 μmole) of polyglutamic acid yielded the following N-terminal sequence:

```
                              *   *       *           *   *   *
H-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-Glu-
  1                   5                       10                  14
```

At the starred positions, the main spot was indeed Glu; in addition, however, faint but distinct spots of Gln were seen. The intensity of the spots decreased gradually and identification ceased to be significant after the first 14 residues. In a second run, on a different preparation, the decrease was even more rapid and only 3 residues could be identified; that time, a

glutamine spot was observed at position 3, in addition to glutamic acid.

When a sample of Gln is submitted to the normal treatment which converts thiazolinones into phenylthiohydantoins, thin layer chromatography reveals that about 50 % of the sample has undergone deamidation. In accordance with this, at positions in proteins where Gln is expected, spots of Glu and Gln of about equal intensity are observed (10). It thus seems that the samples were already partly deamidated when they were submitted to the automatic Edman degradation and in addition that amidation and/or deamidation is random.

DISCUSSION.

The presence of a polyglutamic acid in mycobacterial cell walls has been suggested by the results of Migliore et al. (1) who found large quantities of glutamic acid in the cell wall of pathogenic strains and further substantiated by the work of Acharya (2), Vilkas (3) and ourselves (4).

In the present paper, we describe a method of extraction which allowed us to obtain a rather large molecule of at least 35,000 daltons (based on N-terminal determination) which is insoluble at acid pH's. Elementary analysis led us to postulate that it was partly amidated.

To further analyse this point and to extend our previous results (4) obtained for the polyglutamic acid of BCG cell walls from which we obtained L-glutamic acid oligopeptides which seemed to be α-linked , we resorted to automatic Edman degradation. It allowed us to prove α-linkage for 14 residues and the partial amidation of the extracted polymer. As partial acid hydrolysis of the cell walls was a prerequisite to its extraction, we cannot, for the time being, evaluate the extent of amidation of the native polymer which could have been totally amidated. The reason of its insolubility might be its original size or its linkage to other cell wall components which would be split by the partial acid hydrolysis. The presence in the purified product of small contaminants (see Table 2), which could be remnants of the covalent bridges which link it to other polymers of the cell wall, could in this respect take a particular significance.

One important question is still unanswered: what is the role of the polyglutamic acid present in Mycobacteria cell walls ? Our opinion is that there is a relationship between the virulence of a mycobacterial strain and the

poly-α, L-glutamic acid content in the cell wall. It is not present in the saprophytic strains examined so far , M.smegmatis and M.phlei : it constitutes 8 % in the cell walls of the pathogenic strain M.tuberculosis Brévannes and 2 % in the attenuated M.tuberculosis BCG Pasteur strain.

ACKNOWLEDGMENTS.

We thank Professor E.Lederer for his invaluable advice and constant interest in this work. We are grateful to Dr. Augier (Institut Pasteur, Paris) for providing the cells of M.tuberculosis Brévannes used in this study. We thank E.Tzagarakis for his help in the preparation of the cell walls, A.Yapo for performing the amino-acid analyses and Dr. C.Gros for her advice for the dansylation of N-terminal amino acids. This work was supported in part by grants from the New York Cancer Research Institute and the Fondation pour la Recherche Médicale Française.

REFERENCES.

1- Migliore D., Acharya N. and Jollès P. (1966), C.R.Acad.Sci., (Paris), 263 D, 846-848.
2- Acharya N. (1967) Doctoral Thesis, Paris-Orsay.
3- Vilkas E. and Markovits J. (1972), C.R.Acad.Sci., 275 C, 913-915.
4- Wietzerbin-Falszpan J., Das B., Gros C., Petit J.F. and Lederer E., (1973) Eur. J. Biochem., 32, 525-532.
5- Gros C. and Labouesse B., (1969) Eur. J. Biochem., 7, 463-470.
6- Edman P. and Begg G. (1967), Eur. J. Biochem., 1, 80-91.
7- Herman J., Jollès J., Buss D.H. and Jollès P. (1973) J.Mol.Biol., 79, 587-595.
8- Wietzerbin J., Das B., Petit J.F., Lederer E., Leyh-Bouille M. and Ghuysen J.M. (1974), Biochemistry, 13, 3471-3476.
9- Simons E.R., Fasman G.D. and Blout E.R. (1961), J.Biol.Chem., 236, PC 64-PC 65.
0- Lederer F. (1972) Eur. J. Biochem., 31, 144-147.