ISOLATION AND CHARACTERIZATION OF URIDINE DIPHOSPHATE-N-GLYCOLYLMURAMYL-L-ALANYL-γ-D-GLUTAMYL-MESO-α,α'-DIAMINOPIMELIC ACID FROM MYCOBACTERIUM TUBERCULOSIS

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Received January 9, 1970

Summary: When growing cells of the H37Ra strain of Mycobacterium tuberculosis are exposed to 1.0 mM D-cycloserine, an intracellular peptide-containing nucleotide accumulates. This incomplete precursor for the synthesis of cell wall was isolated and characterized as UDP-N-glycolylmuramyl-L-Ala-γ-D-Glu-meso-DAP.

UDP-N-acetylmuramyl-pentapeptides are precursors for the mucopeptides in bacterial cell walls (1). An incomplete precursor, lacking the C-terminal D-Ala-D-Ala, accumulates in several bacterial species after exposure to D-cycloserine (2-5). Since N-glycolylmuramic acid was discovered recently in the cell walls of Mycobacterium smegmatis (6), the nature of any incomplete precursor that accumulates in mycobacteria would be of interest. Moreover, the knowledge of the structure of this precursor would give us insight into the as yet unknown structure of the peptidoglycan of mycobacterial cell wall.

In this communication, we report that in the presence of D-cycloserine there is an accumulation of UDP-GlycMur-Pep[2] in the H37Ra strain of Mycobacterium tuberculosis.

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[2] Abbreviations: UDP-GlycMur-Pep, UDP-N-glycolylmuramyl-L-Ala-γ-D-Glu-meso-DAP; DAP, α,α' diaminopimelic acid; DNP, 2,4 dinitrophenyl; TMS, trimethylsilyl.

Experimental: Cultures of the H37Ra strain of M. tuberculosis were grown at 37° in a 28-liter capacity fermentor (New Brunswick MicroFerm) in a glycerol-glutamate-salts medium. Two weeks after inoculation, D-cycloserine was added to a final concentration of 1.0 mM; the cells were harvested 24 hr later. The drug treated cells (464 g wet wt) were suspended in 250 ml of 5% Sarcosyl-L (Geigy Industrial Chemicals, Ardsley, N.Y.) - 50% sucrose in 0.01 M Tris, pH 7.5, and allowed to stand 30 min at 0°. The cells were ruptured by the rapid addition of nine volumes of 0.03 M Tris, pH 7.5 and allowed to stand overnight at 0-5°. The supernatant was recovered and deproteinized with perchloric acid. The neutral nucleotide extract was adsorbed onto and then eluted from a charcoal (Nuchar C) column. The nucleotide sample was fractionated on a column of Dowex 1-X8 (Cl^- form, 200-400 mesh, 2 × 26 cm) as described by Nakatani et al. (5). The UDP-GlycMur-Pep fraction was fractionated and desalted on a Bio-Gel P-2 column (100-200 mesh, 2 × 65 cm). The N-acylhexosamine peak (7) was further fractionated by successive paper chromatography in isobutyric-acid-0.5 N NH_4OH (10:6) and n-butanol-acetic acid-water (12:3:5).

Gas chromatographic analysis of the glycolic acid as the TMS derivative (8) was performed on a Barber-Coleman Model 5000 chromatograph using a 72 × 0.17 in. column packed with 15% SE-30 on Anakrom AB. The column was operated at 87° with an argon flow rate of 70 ml per min.

The UDP-GlycMur-Pep was completely hydrolyzed in 6 N HCl at 100° for 18 hr. Hydrolysis to release the amino sugar and glycolic acid was done in 2 N HCl at 110° for 4 hr. N-glycolylmuramyl-tripeptide was obtained by hydrolyzing UDP-GlycMur-Pep in 0.05 N HCl at 100° for 20 min and purified by paper chromatography.

Optical configurations of alanine and glutamic acid were determined

by specific enzymatic methods (9, 10). Hydrazinolysis was performed on the N-glycolylmuramyl-tripeptide by the method of Ghuysen *et al.* (11).

Results: The purified UDP-GlycMur-Pep behaved as a homogenous compound on paper chromatography in five solvent systems and on paper electrophoresis at pH 3.6 and 6.5. About 3 μmoles of the UDP-GlycMur-Pep were obtained from 100 g (wet wt) of cells. In the absence of D-cycloserine, the yield of UDP-GlycMur-Pep was less than 0.4 μmole per 100 g of cells. The absorption spectrum of the purified UDP-GlycMur-Pep was identical to that of uridine at both pH 7.0 and 11. After hydrolysis of UDP-GlycMur-Pep in 0.01 N HCl at 100° for 10 min, UDP and N-glycolylmuramyl-tripeptide were released. Hydrolysis of the parent compound in 1.0 N HCl at 100° for 10 min released UMP.

The amino sugar in UDP-GlycMur-Pep was identified as muramic acid by paper chromatography in two solvent systems and by the characteristic 3.4 fold increase in absorbance at 505 *nm* in the Elson-Morgan reaction after 24 hr (12). The amino acids present in UDP-GlycMur-Pep were DAP, glutamic acid and alanine.

The purified UDP-GlycMur-Pep consistently gave low (about 60%) color yields for muramic acid when assayed for N-acetylhexosamine (7). The organic acid present in UDP-GlycMur-Pep corresponded to authentic glycolic acid (CalBiochem) on paper chromatography (13). By gas-liquid chromatography, the TMS derivative of the organic acid in UDP-GlycMur-Pep corresponded to TMS glycolate. The TMS derivative of lactic acid has the same retention time, under these conditions, as TMS glycolate, however, lactic acid was excluded by the paper chromatographic systems. No other volatile organic acids were found.

The alanine and glutamic acid in UDP-GlycMur-Pep were found to

Table I. Molar Composition of UDP-GlycMur-Pep

Substance	Molar ratio		
	Calculated from data		Theoretical value
Uridine[a]	1.00	1.00[f]	1
Total phosphorus[b]	1.91	--	2
Acid-labile phosphate[c]	0.94	--	1
Muramic acid[d]	0.83	1.06	1
Alanine	--	1.04	1
Glutamic acid	--	1.02	1
DAP	--	1.16	1
Glycolic acid[e]	1.37	--	1

[a]The uridine content was calculated by using the molar extinction coefficient of 9.9×10^3 at $A_{260\ nm}$.

[b]Total phosphorus was assayed by the method of Bartlett (15).

[c]Inorganic orthophosphate liberated by heating the sample in 1 N HCl at 100° for 20 min and assayed by method of Fiske and SubbaRow (16).

[d]Muramic acid was assayed by the method of Elson-Morgan (12).

[e]Determined by gas-liquid chromatography of the TMS derivative of the acid hydrolyzed sample.

[f]The data for this column were obtained from the analysis of the sample in an amino acid analyzer after hydrolysis in 6 N HCl at 100° for 6 hr. This analysis was kindly performed for us by Dr. Donald Tipper.

be the L- and D-isomers respectively. The di-DNP derivative of the DAP obtained from UDP-GlycMur-Pep co-chromatographed with authentic di-DNP-meso-DAP (Mann Research Lab) on thin-layer chromatography (9, 14). The analytical data on the purified UDP-GlycMur-Pep shown in Table I established the molar ratios of the components.

Dinitrophenylation of the N-glycolylmuramyl-tripeptide followed by hydrolysis and thin-layer chromatography (9) gave a single DNP derivative, mono-DNP-meso-DAP, with an 88% yield. Hydrazinolysis of N-glycolylmuramyl-tripeptide revealed meso-DAP, alanine hydrazide and the γ hydrazide of glutamic acid. Thus the C-terminal position is occupied by meso-DAP and the γ-carboxyl group of glutamic acid is involved in the peptide linkage.

The N-glycolylmuramyl-tripeptide was partially hydrolyzed in 2.5 N HCl at 100° for 30 min. The degradation products were separated by paper chromatography and identified. A dipeptide fragment (L-Ala-D-Glu) and a tripeptide fragment (L-Ala-D-Glu-meso-DAP), both containing N-terminal alanine, were obtained.

Discussion: The incomplete precursor of M. tuberculosis has been established to be UDP-GlycMur-Pep. This structure is novel because of the presence of the N-glycolyl group. The tripeptide of this incomplete precursor is similar to the tripeptide, Ala-Glu-DAP, isolated from the peptidoglycan of M. smegmatis by Petit et al. (17). The tetrapeptide, Ala-Glu-DAP-Ala, which was also isolated from M. smegmatis, is consistent with the expected peptide structure of the complete precursor. The peptide subunit and the cross-linking bridge of the peptidoglycan in the mycobacteria appear to be Type A and Type I, respectively when classified according to Ghuysen (18).

Migliore and Jolles (19) proposed a tentative structure of the peptidoglycan in Wax D and suggested that it is identical to the peptidoglycan of the cell wall. David et al. (20) showed that the synthesis of Wax D in M. tuberculosis is inhibited by D-cycloserine. Thus the same nucleotide compound could also be the precursor to the synthesis of Wax D. Investigation of this role is continuing.

Acknowledgements: We thank Mrs. Emma Lee Armstrong for her excellent technical assistance. We also thank Dr. Donald Tipper (University of Wisconsin) for advice in many phases of this work. This investigation was supported in part, by Research Grant 5 RO1 AI01816-11 from the National Institute for Allergy and Infectious Diseases, U.S.P.H.S.

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