MANNOLIPID SYNTHESIS IN A CELL-FREE SYSTEM OF *MYCOBACTERIUM SMEGMATIS*

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Received 27 August 1971

1. Introduction

Mycobacteria contain unusual and specie specific phosphatidyl *myo*-inositol oligomannosides (PI-M_x) where the number of mannose units (x) may vary from one to five [1]. The biosynthesis of these mannophospholipids have been studied only in the cell-free systems of the H37Ra strain of *Mycobacterium tuberculosis* and *Mycobacterium phlei* [2–6]. The major product of the mannose transfer reaction in the *M. tuberculosis* has been shown to be mannosyl-1-phosphoryl-decaprenol (MPD) [3]. One of the most puzzling aspects of these studies is that only the synthesis of PI-M_2 can be unequivocally demonstrated. The synthesis of the higher homologues of these mannophospholipid series (PI-M_3, PI-M_4, and PI-M_5) have not been shown. There is also some doubt as to whether the synthesis of PI-M_1 occurs. Thus progress has been hampered by the apparent lack of a suitable enzyme system to study this mannose transfer reaction in mycobacteria.

In this paper we report that the cell-free particulate system of *Mycobacterium smegmatis* synthesizes ^{14}C-mannosyl-phosphoryl-lipid which appears to be related to MPD as well as all of the known series of the labelled PI-M_x from the substrate GDP-D-mannose-^{14}C. Thus the cell-free system of *M. smegmatis* is uniquely suited to the study of the biosynthesis of the PI-M_x.

2. Materials and methods

GDP-D-mannose-^{14}C was purchased from New England Nuclear. MPD was prepared from *M. tuberculosis* according to previously described procedure [3]. Phosphatidyl *myo*-inositol (plant) was purchased from Applied Science.

The preparation of α-glyceryl-phosphoryl-*myo*-inositol di- and pentamannosides from *M. tuberculosis* were previously described [2, 7]. The α-glyceryl-phosphoryl-*myo*-inositol tri- and tetramannoside were similarly obtained from the phospholipid fraction of the whole cells of *M. tuberculosis*. Preparative paper chromatography of the crude α-glyceryl-phosphoryl-*myo*-inositol di- and pentamannoside fractions obtained from the DEAE-Sephadex column [7] yielded the tri- and tetramannoside derivatives whose chemical analysis showed the ratio mannose: phosphorus:glycerol:*myo*-inositol to be 3.1:1.0:1.2:0.8 and 4.2:1.0:2.0:0.8 respectively. The above purified compounds were dephosphorylated according to the method of Ballou et al. [8] to obtain the corresponding derivatives of the *myo*-inositol di-, tri-, tetra-, and pentamannosides. Each of the purified dephosphorylated derivatives was analyzed for total phosphorus, *myo*-inositol, and mannose contents as previously described [7]. The preparation of *myo*-inositol monomannoside is described elsewhere [2].

The growth and harvest procedures of the H37Ra strain of *M. tuberculosis* are described elsewhere [9]. A 105,000 *g* residue fraction of *M. tuberculosis* was obtained by a published procedure [3]. *M. smegmatis* ATCC 607 was grown in glycerol–glutamate–salt medium at 37° for 48 hr on a rotary shaker. Cells were harvested by filtering and washed with water. Ten grammes wet weight of cells were suspended in 50 ml of 0.01 M Tris-HCl, pH 7.5 and subjected to sonic oscillation with a Branson probe sonifier at full power for a total of 10 min at 0°. Cell debris was removed

by centrifuging at 10,000 *g* for 30 min. The supernatant was centrifuged at 105,000 *g* for 90 min to obtain the membrane fraction which contained the transmannosylase enzyme. This enzyme was suspended in 0.01 M Tris-HCl, pH 7.5 and dialyzed against the same buffer for 120 min. The transmannosylase enzyme activity was assayed by a previously described method [3]. The mannophospholipids were deacylated by the method of Dawson [10]. The water-soluble fraction was passed through a small Dowex 50 (H^+) column and neutralized with NH_4OH.

The reaction mixture for the synthesis of ^{14}C-mannolipids contained 50 μmole Tris-HCl, pH 8.0; 10 μmole $MgCl_2$; 50 nmole GDP-D-mannose-^{14}C (1.1×10^6 dpm); 11 mg of 105,000 *g* residue fraction from *M. smegmatis* in a final volume of 1.50 ml. The reaction mixture was incubated at 37° for 60 min. The ^{14}C-mannolipids were extracted three times with 4.0 ml aliquots of chloroform–methanol (2:1); the pooled extract was washed once with 4.0 ml of 1% NaCl solution and dried. The yield of the labelled mannolipid product was 309,000 dpm.

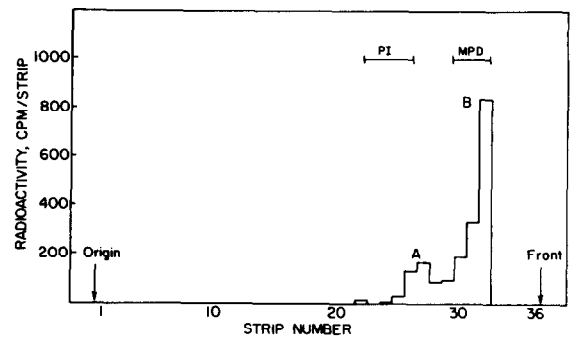

Fig. 1. Thin-layer chromatography of the alkali-stable ^{14}C-mannolipid. Alkali-stable ^{14}C-mannolipids were obtained after deacylation of the enzymatically synthesized total ^{14}C-mannolipid product. An aliquot of this fraction (3850 cpm) was chromatographed on a Silica Gel G plate in chloroform–methanol–acetic acid–water (30:15:4:2) along with phosphatidyl *myo*-inositol (PI) and mannosyl-1-phosphoryl-decaprenol (MPD). One-half cm strips were scraped into counting vials and counted in the toluene scintillator fluid.

3. Results and discussion

When a particulate cell-free fraction of the *M. smegmatis* was incubated in the presence of GDP-D-mannose-^{14}C, the radioactive mannose was transferred to the lipid fraction. Like the transmannosylase system of the H37Ra strain of *M. tuberculosis* [3], the synthesis of the mannolipid was dependent on both time and protein concentration. The specific enzyme activity of the cell-free system of the *M. smegmatis* was consistently higher than that of the *M. tuberculosis* by a factor of about 10 to 15.

Upon deacylation of 300,000 dpm of the mannolipid product the distribution of radioactivity was as follows: 90% (194,000 dpm) in the alkali-stable fraction and 10% (21,200 dpm) in the alkali-labile fraction. The recovery radioactivity was 73%. The alkali-stable fraction was examined by thin-layer chromatography (fig. 1). The major radioactive product (peak B) was chromatographically identical with authentic MPD. A minor alkali-stable product (peak A) appeared to correspond to a minor product found in *M. tuberculosis* [3]. The major alkali-stable lipid exhibited both chromatographic and hydrolytic characteristics that were identical to those described for the MPD isolated from *M. tuberculosis* [3]. From these tests we suggest that the major alkali-stable product is ^{14}C-mannosyl-1-phosphoryl-lipid. As in the MPD, the lipid moiety is thought to be a polyisoprenol.

The alkali-labile fraction obtained after deacylation was analyzed by paper chromatography. Fig. 2A shows the presence of α-glyceryl-phosphoryl-*myo*-inositol- di-, tri-, tetra-, and pentamannoside-^{14}C. The radioactive peak M is thought to be the α-glyceryl-phosphoryl-*myo*-inositol monomannoside. These derivatives would correspond to the phosphatidyl *myo*-inositol-mono-, di-, tri-, tetra-, and pentamannoside (PI-M_1 through PI-M_5). The deacylated lipid (13,600 dpm) was dephosphorylated to yield 8500 dpm of the neutral derivative (62%). An aliquot of the dephosphorylated sample was then chromatographed on paper. Using three solvent systems of *n*-butanol–ethanol–water (2:1:1), *n*-butanol–pyridine–water (10:3:3), and *n*-butanol–acetic acid–water (12:3:5), identical results were obtained (fig. 2B). The presence of *myo*-inositol mono-, di-, tri-, tetra-, and pentamannoside-^{14}C were shown by paper chromatography. These are the expected products to be obtained from the mannophospholipids PI-M_1 through PI-M_5. The major alkali-labile mannolipid product was shown to be PI-M_4 whereas the PI-M_2 was found to be the minor product. The *M. tuber-*

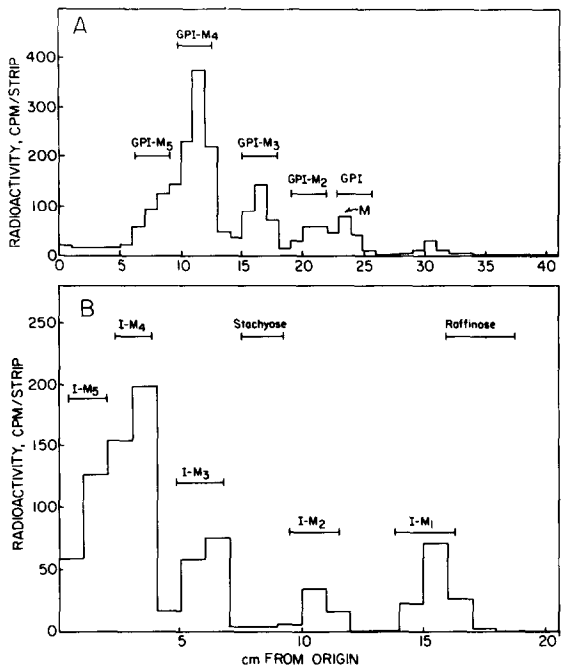

Fig. 2. Paper chromatography of the deacylated (A) and dephosphorylated (B) labelled mannophospholipids synthesized enzymatically from GDP-D-mannose-^{14}C. The ^{14}C-mannophospholipids were deacylated and 2700 cpm of the water-soluble product was chromatographed using the solvent *iso*-propanol–water (3:1) in ammonia atmosphere (fig. 2A) along with α-glyceryl-phosphoryl-*myo*-inositol (GPI); α-glyceryl-phosphoryl-*myo*-inositol-dimannoside (GPI-M_2), trimannoside (GPI-M_3), tetramannoside (GPI-M_4), and pentamannoside (GPI-M_5). The deacylated product was dephosphorylated and chromatographed (sample size 1580 cpm) using the solvent *n*-butanol–ethanol–water (2:1:1) (fig. 2B) along with *myo*-inositol-monomannoside (I-M_1), dimannoside (I-M_2), trimannoside (I-M_3), tetramannoside (I-M_4), and pentamannoside (I-M_5); raffinose and stachyose. One cm strips were cut out and assayed for radioactivity using the scintillation spectrometer. All of the radioactivity present in both deacylated and dephosphorylated samples appear in these figures.

culosis [2, 3] and the *M. phlei* [4–6] cell-free systems can only synthesize PI-M_2. The particulate cell-free system of *M. smegmatis* thus appears to be an appropriate system to study the biosynthesis of the phosphatidyl *myo*-inositol oligomannosides.

Acknowledgement

This research was supported in part by Grant GB-13598 from the National Science Foundation.

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