TRANSMETHYLATION TO α-GLYCEROL PHOSPHATE; A POSSIBLE PRECURSOR IN THE FORMATION OF 6-O-METHYL-D-GLUCOSE IN MYCOBACTERIUM TUBERCULOSIS

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The occurrence in mycobacteria of a number of related polysaccharides containing 6-O-methyl-D-glucose is the subject of several recent studies.[1-3] 6-O-methyl-D-glucose is a likely precursor to the polysaccharides containing 6-O-methyl glucose as well as other methylated sugars.[4] In this report we show that cell-free extracts of the H37Ra strain of Mycobacterium tuberculosis contain an enzyme which catalyzes the transfer of the methyl group of S-adenosyl methionine to α-glycerol phosphate forming 3-methoxy 1, 2 propanediol (α-glyceryl methyl ether, α-GME). The methyl group is thus introduced at the triose level for incorporation into hexoses and other sugars.

Materials and Methods

S-Adenosyl methionine (SAM) and (methyl ^{14}C)-SAM were obtained from Calbiochem, and (DL)3-methoxy 1, 2 propanediol (α-glyceryl methyl ether, α-GME) from K and K Laboratories.

Cell-free extracts of 21-day-old cultures of the H37Ra strain of Mycobacterium tuberculosis were prepared as previously described.[5] The preparation of the transmethylase system was carried out at 2C. The soluble fraction of the cell-free extract was fractionated at pH 7 with ammonium sulfate. The fraction precipitating at 0.40 saturation was discarded; the fraction precipitating between 0.40 and 0.60 saturation was recovered after centrifugation and dissolved in a minimal quantity of water.

The transmethylation mixture contains $MgCl_2$, 2 μmoles; NaCl, 70 μmoles; SAM, 0.25-0.5 μmole ($10\text{-}90 \times 10^3$ cpm ^{14}C); glycine buffer, pH8, or phosphate or pyrophosphate buffer, pH 7.5 (10-25 μmoles), 1-2 mg of pro-

tein, and α-glycerol phosphate (or endogenous acceptor, see below) in 0.8 ml final volume.

Phosphate or pyrophosphate, while increasing the ^{14}C in the blank (^{14}C incorporation into neutral material), is necessary to maintain activity during dialysis of partially purified transmethylase. Dialysis for 18 hours at 2C (0.02 M phosphate or pyrophosphate, pH 7.5) removes the majority of endogenous acceptor otherwise carried along in the purification; however, if all the endogenous acceptor is removed by extensive dialysis (48 hours) the enzyme becomes inactive.

Results

When SAM was incubated with crude undialyzed cell-free extract a neutral labeled product was formed which chromatographed on paper with sugars of polarity lowered by two methyl groups, such as 2-O and 3-O methyl rhamnose. The molecular weight of the product, however, was considerably less than that of hexoses, as determined by comparing the elution volume from Biogel P_2 with that of 2-O-methyl rhamnose, glucose or mannose. These results suggested a triose with one methyl group instead of a hexose with two methyl groups.

The low molecular weight product was also obtained in the presence of SAM and crude dialyzed cell-free extract if deproteinized cell-free extract (see below) was also present. The product did not form if deproteinized cell-free extract was omitted.

Isolation of an endogenous methyl acceptor: The crude soluble cell-free extract is deproteinized by heating at 95-100C for 20 minutes, cooling and centrifuging at 30,000 g for 20 minutes. The supernatant is brought to pH 2 with HCl and centrifuged again. The pH of the supernatant is brought to 5.5 with

KOH, or to 7.5 if assayed at this step.

The pH 5.5 supernatant is passed over a 2 × 4 cm column of washed and outgassed Nuchar-C. Elution is with water; the first fraction is collected until ultraviolet absorbing material begins to appear in the effluent. This fraction is concentrated to 3 ml by evaporation. The crystalline suspension which usually forms is removed by centrifugation. The soluble fraction yields about 40 mg of a low molecular weight phosphorylated substance identified as (L) α-glycerol phosphate (as determined by (L) α-glycerol phosphate dehydrogenase) along with 3 mg of saccharide material (as determined by sulfonated resorcinol)[6] from 200 ml of starting material.

The concentrated effluent from the charcoal step is active as a methyl acceptor. DL-α-Glycerol phosphate subsitutes for the endogenous acceptor (Table I). Other trioses were inactive. The results of table I show that α-glycerol phosphate stimulates incorporation of ^{14}C methyl groups into neutral material.

Isolation and Identification of Product: Transmethylation was carried out as above, with 2 μmoles DL-α-glycerol phosphate and 3 mg protein (dialyzed 40-60% ammonium sulfate fraction). After incubation for one hour at 37C the mixture was deproteinized with 50 μl 2N perchloric acid. The deproteinized mixture was diluted with 1.5 ml ethanol, neutralized with 100 μl of 1N KOH and centrifuged. The supernatant was passed through a 1 × 4 cm column of Dowex 50 (H^+) and neutral compounds were eluted with 10 ml ethanol-water 1:1. Part of the effluent was mixed with 5 ml chloroform and treated with 5 grams of anhydrous sodium carbonate. The supernatant was co-distilled with 5 ml of 3-methoxy-1,2 propanediol. Four fractions containing the constant boiling α-GME (117C, 15 mm) were taken. Each fraction had the same

Table I

Acceptor		Neutral product formed	Δ mμmoles
	(μmoles)	(mμmoles)	
None	~	3.4	~
α Glycerol-P	2.0	6.6	3.2
Glycerol	5.0	3.9	0.5
Glyceraldehyde	5.0	4.2	0.7
Glyceraldehyde 3-P	2.0	3.9	0.5
Dihydroxyacetone	5.0	3.2	-0.2
None	~	22.2	~
α Glycerol-P	2.5	25.3	3.1
α Glycerol-P	6.0	24.6	2.4
β Glycerol-P	2.5	23.2	1.0
β Glycerol-P	6.0	22.0	-0.2

Transmethylation conditions: SAM as limiting reagent (160 mμmoles), including 12,500 cpm of ^{14}C SAM. In the first six sets of incubations, each tube contained 3 mg crude protein dialyzed overnight in 0.01 M glycine, pH 8.0. In the remaining five incubations, each tube contained 1.5 mg protein from a 40-60% ammonium sulfate fraction and 5 μmoles phosphate buffer, pH 7.5, resulting in a larger blank formation of neutral ^{14}C-containing material. Other conditions same as in text.

Table II

Starting material, counts (^{14}C-SAM)	α-Glycerol-P added	^{14}C in Neutral Counts*		
		Total	Co-distilling with α-GME	As Methoxy-acetaldehyde
(cpm)	(μmoles)	(cpm)	(cpm)	(cpm)
90,000	0	7990	232	240
90,000	2.0	8950	1056	952

* Includes 5-thiomethyl 5-deoxyribose and ^{14}C-methanol from the hydrolysis of ^{14}C-SAM.

The reaction mixtures include SAM, 0.5 μmole and 3 mg protein (40-60% ammonium sulfate fraction dialyzed overnight in 0.02 M phosphate, pH 7.5); other conditions as given in text.

proportion of radioactive material relative to recovery of α-GME. The four fractions were combined and esterified with propionic anhydride in pyridine. After washing excess propionic acid and pyridine from the α-GME dipropionate, distillation was carried out at 130C (15 mm Hg). Again, the ratio of labeling to carrier material is constant for the four fractions.

When the anhydrous effluent from Dowex 50, without prior distillation, is combined with carrier α-GME and treated directly with excess propionic anhydride and pyridine, the results are similar.

In order to distinguish between labeled α-GME and β-GME (2-methoxy-1,3 propanediol), part of the neutral product was subjected to periodate cleavage. The effluent from the Dowex 50 column was mixed with chloroform and dehydrated as before. Three ml of dioxane were added and the mixture was evaporated under reduced pressure (30C) to 1.0 ml. This step is necessary to remove any ^{14}C-methanol formed in the presence of the transmethylase.

The mixture was diluted with 2.5 ml ethanol and an excess (100 mg) of carrier α-GME was added. Periodic acid (0.05 mmole in 2.5 ml H_2O) was added and the mixture was incubated at room temperature for 15 minutes. The mixture was treated with excess barium carbonate to remove iodic acid and then distilled with 4 ml n-butanol. Methoxy acetaldehyde in the distillate was estimated by oxidation to methoxy acetic acid with chromic acid in 25% H_2SO_4 at room temperature, and measuring the color formed with chromotropic acid reagent.[7] Upon heating the reaction mixture at 100C for 25 minutes, methoxyacetic acid is cleaved to glycolic acid which reacts with the chromotropic acid.[8]

All the ^{14}C-containing neutral product that co-distills with carrier α-GME is recovered after periodate oxidation as ^{14}C methoxy acetaldehyde

(Table II). β-GME does not react with periodate under these conditions. Table II shows that the formation of α-GME depends on α-glycerol phosphate. Most of the ^{14}C content of the blank in the assay is dependent on phosphate, but is independent of acceptor. The ^{14}C-containing compound is volatile, distills with methanol, and on propionylation, with methyl propionate, establishing it as ^{14}C methanol. Some of the ^{14}C content of the blank is non-volatile and arises independently of phosphate and of protein. It is most likely the neutral hydrolysis product, 5-thiomethyl, 5-deoxy ribose. The stimulation of enzymatic hydrolysis of SAM by phosphate (Tables I and II) may give a clue to the mechanism of the transmethylation to α-glycerol phosphate. The fate of the phosphate group of α-glycerol phosphate is presently under investigation

DISCUSSION

Attempts to isolate (^{14}C) 6-O-methyl-D-glucose from an analagous transmethylation of ^{14}C-SAM to glucose 6-phosphate were not successful.

α-GME may therefore be the first O-methylated compound in the biosynthesis of 6-O-methyl glucose. We have found (unpublished) that α-GME is a good substrate for the TPN-dependent glycerol dehydrogenase prepared from M. tuberculosis.[5] From the triose and methyl-triose level, the formation of methyl hexose becomes possible.

The mycobacterial transmethylation system described above may differ from that of Okuda, Suzuki and Suzuki,[9] who have suggested that a nucleotide of a keto sugar and SAM are starting materials for introduction of an O-methyl group into the hexose ring. We cannot as yet exclude the possibility of a cofactor in this reaction.

The precedents for direct O-methylation from SAM in which catechol derivatives act as acceptors are outlined by Schlenk.[10]

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