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A PROTEIN-BOUND PHOSPHORYLATED PRODUCT AS AN INTERMEDIATE IN THE BIOSYNTHESIS OF *O*-METHYL GLYCEROL BY *MYCOBACTERIUM TUBERCULOSIS*

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SUMMARY

1-*O*-methyl *sn*-glycerol 3-phosphate bound to a protein has been identified as the probable intermediate in the mycobacterial transmethylation reaction leading to the formation of 1-*O*-methyl glycerol from *S*-adenosyl methionine and *sn*-glycerol 3-phosphate. Acid treatment of the partially purified protein-bound product appears to release 1-*O*-methyl *sn*-glycerol 2:3-cyclic phosphate. Our hydrolysis studies indicate that the 1-*O*-methyl glycerol is bound to the protein *via* a phosphodiester linkage.

INTRODUCTION

Cell-free preparations of the H37Ra strain of *Mycobacterium tuberculosis* contain a soluble transmethylation system in which *sn*-glycerol 3-phosphate is the acceptor of a methyl group from *S*-adenosyl methionine and 1-*O*-methyl glycerol is the product[1].

Recent work in our laboratory (unpublished) showed that in addition to *rac*-1-*O*-methyl glycerol (1-methoxy-2,3-propanediol or α-glycerol methyl ether), another product was formed by the cell-free transmethylase system of *M. tuberculosis* which was apparently released by a mild acid treatment. In this paper we describe the isolation and characterization of a labeled anionic product of the enzymatic transmethylation reaction and present evidence that it was derived from a protein-bound form of 1-*O*-methyl *sn*-glycerol 3-phosphate.

MATERIALS AND METHODS

The H37Ra strain of *M. tuberculosis* was grown in 20-l batches in a glycerol–alanine–salts medium to the end of the log phase. Cell-free extracts were prepared as described previously[2] and dialyzed for 48 h against 30 mM phosphate (pH 7.0)–7 mM $MgCl_2$. Glycerokinase of *Candida mycoderma* was obtained from Calbiochem; the

Abbreviation: BBOT, 2,5-bis-[2-(5-*tert*-butylbenzoxazolyl)] thiophene.

stabilizing glycerol present in the preparation was removed by dialysis against 10 mM *rac*-1-*O*-methyl glycerol. Purified *rac*-1-*O*-methyl glycerol was prepared by double vacuum distillation of commercial *rac*-1-*O*-methyl glycerol at 108 °C. BBOT-toluene scintillation fluid for counting radioactivity on thin-layer chromatographic strips consisted of 0.4% diphenyl oxazole (PPO) and 0.1% 2,5-bis-[2-(5-*tert.*-butylbenzoxazolyl)] thiophene (BBOT) from Packard Instruments Co. in A.R. toluene. The source of other materials and reagents has been described[1].

Preparation and characterization of 1-O-methyl sn-glycerol 3-phosphate

1-*O*-methyl *sn*-glycerol 3-phosphate was prepared by phosphorylating *rac*-1-*O*-methyl glycerol with ATP in the presence of *Candida mycoderma* glycerokinase[3]. The ADP formed in the reaction was transphosphorylated to ATP with creatine phosphate in the presence of creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2)[4]. The reaction mixture contained ATP 125 μmoles; creatine-*P*, 125 μmoles; $MgCl_2$, 250 μmoles; Tris–HCl, pH 7.5, 2 mmoles; *rac*-1-*O*-methyl glycerol, 10 mmoles; glycerokinase, 5 mg; and creatine kinase, 5 mg; in a total volume of 60 ml. Incubation was carried out at 25 °C for 7 h. The reaction was stopped by addition of excess (5 g) Dowex 50 (H^+). The resin was removed; the supernatant solution was treated with an excess (2 g) of barium carbonate. The supernatant solution from this step was treated with sufficient ammonium carbonate to precipitate all of the dissolved barium and centrifuged once more. This final supernatant was passed over a DEAE-Sephadex (carbonate) column. The column was washed with water. The product was eluted from the column with 0.5 M ammonium carbonate (pH 8). The ammonium carbonate was removed by three cycles of evaporation–resolution in water. 25 μmoles of product were recovered. The product migrates as a single spot with an R_F of 0.64 on thin-layer cellulose strips (Bakerflex) in *n*-propanol–NH_4OH–water (6:3:1, by vol.)[5]. Authentic *sn*-glycerol 3-phosphate in the same system shows an R_F of 0.38. Color development was carried out with the iron–sulfosalicylate spray of Wade and Morgan[6] which is specific for phosphates.

The glycerokinase is known to phosphorylate glycerol exclusively in the 3-position[7]. Similarly we obtained evidence that the phosphorylated *rac*-1-*O*-methyl glycerol was 1-*O*-methyl *sn*-glycerol 3-phosphate. The free hydroxyl group of the product was oxidized in acid permanganate at 37 °C (ref. 8). After the removal of excess permanganate with SO_2 the phosphate group was then hydrolyzed at 100 °C for 20 min in 0.5 M H_2SO_4 (ref. 9). The product of this oxidation reaction gave a positive test for a reducing sugar with the Tollen's reagent[10] which suggested that the product must be *O*-methyl dihydroxyacetone. This is the expected product if the 3-position of 1-*O*-methyl glycerol is phosphorylated. If the phosphate group were in the 2-position, this procedure would have yielded 3-*O*-methyl glyceric acid 2-phosphate and 3-*O*-methyl glyceric acid, neither of which would react with Tollen's reagent.

The amount of 1-*O*-methyl glycerol 3-phosphate obtained from the glycerokinase reaction was determined by measuring the inorganic phosphate formed (Fiske–SubbaRow)[11] in a chromic acid oxidation procedure. The Cr^{3+} formed in the oxidation was determined spectrophotometrically in order to obtain the ratio Cr^{3+}/phosphate. The experimental result, 5.8 moles Cr^{3+}/mole phosphate agrees well with the theoretical value of 5.5 for the structure of 1-*O*-methyl glycerol 3-phosphate.

Assay for the synthesis of the anionic product of the enzymatic transmethylation

The reaction mixture contained $MgCl_2$, 1.5 μmoles; ammonium bicarbonate, 2 μmoles; EDTA, 0.5 μmole; phosphate, 4–9 μmoles; glycerol 1-phosphate, 0.1 μmole; ^{14}C-labeled *S*-adenosyl methionine, 0.2 μmole; (final pH of mixture: 7.0) and 1 mg of protein in a total volume of 200 μl. The reaction mixtures were incubated at 37 °C for 30 to 60 min. An aliquot, usually 50 μl, of the reaction mixture was added to 100 μl of 0.02 M $HClO_4$ in ethanol. After 15 min the mixture was centrifuged; the clear supernatant was passed over a 2.0 mm × 20 mm column of Dowex 50 (H^+). Neutral and anionic counts were washed through with 1.0 ml of 50% ethanol. An aliquot of the combined effluent–wash was evaporated to dryness and dissolved in 0.07 ml of 50% ethanol. 50 μl were spotted onto a 30 mm × 6 mm strip of DEAE paper suspended in a current of warm air. The strip was washed free of neutral materials by repetitive dipping into distilled water; four changes of distilled water were used. The strip was dried and the radioactivity determined using a scintillation spectrometer with BBOT–toluene scintillation fluid (see 1st paragraph of Materials and Methods).

RESULTS

Initial studies on the anionic labeled product

The synthesis of an anionic product of transmethylation can be detected by treating the reaction mixture with dilute acid at room temperature and counting the radioactivity of the anionic isolate. Since the yield of the anionic product was generally low, various studies were carried out to try to increase it. We found that under optimal conditions the concentration of the product approached about 2 nmoles per reaction mixture. There was direct relationship between protein concentration and amount of anionic product formed. Initial studies were made on the anionic product without further isolation from the cation-exchange treated transmethylation mixtures. These mixtures had both anionic and neutral radioactive material. The acidic effluents from the Dowex 50 column were neutralized with sodium bicarbonate. Labeled 1-*O*-methyl glycerol was removed by adding 5 ml of authentic *rac*-1-*O*-methyl glycerol and distilling under reduced pressure as previously described[1]. The residue which contained only the nonvolatile [^{14}C]methyl compounds (1080 dpm) was divided into several portions for degradation studies.

One aliquot (270 dpm) was treated with *Escherichia coli* phosphomonoesterase under conditions which gave complete hydrolysis of glycerol 3-phosphate. This treatment yielded 54 counts (20%) volatilizing with *rac*-1-*O*-methyl glycerol. The residue of the same sample was then subjected to acid hydrolysis with 2 M HCl at 100 °C for 24 h. No labeled 1-*O*-methyl glycerol distilled over after this treatment. Evidently the ether linkage is cleaved and the resulting methanol volatilized; under the same conditions 30% of the 1-*O*-methyl glycerol ether groups are cleaved. Hydrolysis of another aliquot (270 dpm) in 1 M NaOH at 100 °C for 24 h gave a 26% yield of 1-*O*-methyl glycerol. Further treatment of the alkaline hydrolysis residue with wheat germ phosphomonoesterase yielded another 50% of 1-*O*-methyl glycerol.

Another portion (400 dpm) of the nonvolatile [^{14}C]methyl product was heated with 3,5-dinitrobenzoyl chloride in pyridine[12]. This treatment yielded a derivative that passed through a Sephadex G-10 column with a partition coefficient (K_{av})[13] of

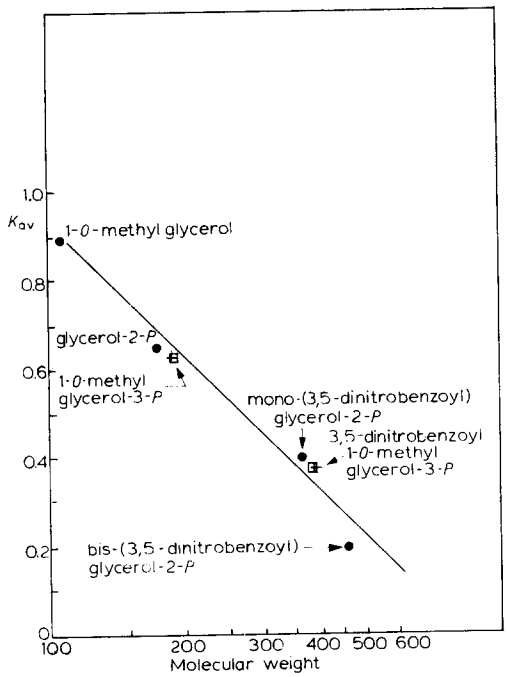

Fig. 1. A semilog plot of K_{av} *vs* molecular weight on Sephadex G-10 column. Abscissa: K_{av} partition coefficient $(V_e - V_0)/(V_t - V_0)$ where V_e elution volume; V_t bed volume; V_0 void volume of standards. Ordinate: ●, molecular weight (each standard indicated next to its plot); +, molecular weights of unknowns; □, theoretical molecular weights of structures assigned to unknown.

0.38 which can be compared to the theoretical value (0.38) for that of 3,5-dinitrobenzoyl glycerol methyl ether phosphate on a semilog plot of K_{av} *vs.* molecular weight (Fig. 1). Underivatized material gave a K_{av} value of 0.62 which can be compared to the theoretical value of 0.61 for *X*-1-*O*-methyl glycerol 3-phosphate. Thus the size of the anionic product and its derivative agrees well with that of 1-*O*-methyl glycerol 3-phosphate and its derivative.

Further studies of the nature of the anionic labeled product

In order to obtain a sufficient amount of the anionic labeled compound for further study the transmethylation reaction mixture was scaled up 100-fold. After the Dowex 50 step the mixture was evaporated to remove ethanol. The residue was dissolved in water and applied to a 1 cm × 5 cm column of DEAE-Sephadex (free base form)*. The loaded column was washed with water. The anionic product was eluted with 5 ml of 3 M NH_4OH. From an initial 15 μmoles of ^{14}C- labeled *S*-adenosyl methionine ($5.8 \cdot 10^6$ dpm) and 100 mg protein, 90 nmoles (34 000 dpm) of product was recovered.

* In contrast to the divalent 1-*O*-methyl *sn*-glycerol 3-phosphate, most of the monovalent cyclic phosphate passes through the DEAE-Sephadex (carbonate); accordingly, the exchanger was used in its free base form.

A small amount (300 dpm) of purified anionic labeled product was mixed with 20 μl of standard 1-*O*-methyl *sn*-glycerol 3-phosphate (100 nmoles) and pipetted along a 10-mm streak onto a Bakerflex thin-layer cellulose coated strip. The chromatogram was run in *n*-propanol–conc. NH_4OH–water (6:3:1, by vol.)[5]. The 1-*O*-methyl *sn*-glycerol 3-phosphate spot was visualized with iron–sulfosalicylic acid spray[6]. The spot was traced and the chromatogram was cut into 0.25-inch strips. The radioactivity of the strips was counted in BBOT–toluene scintillation fluid. The center of the radioactivity profile (anionic labeled product) travels 15% faster than the center of the 1-*O*-methyl *sn*-glycerol 3-phosphate spot as shown in Fig. 2a. Another portion of the purified anionic labeled product (300 dpm) was hydrolyzed in 1 M NaOH at room temperature for 20 h[14]. It was mixed with the standard 1-*O*-methyl *sn*-glycerol

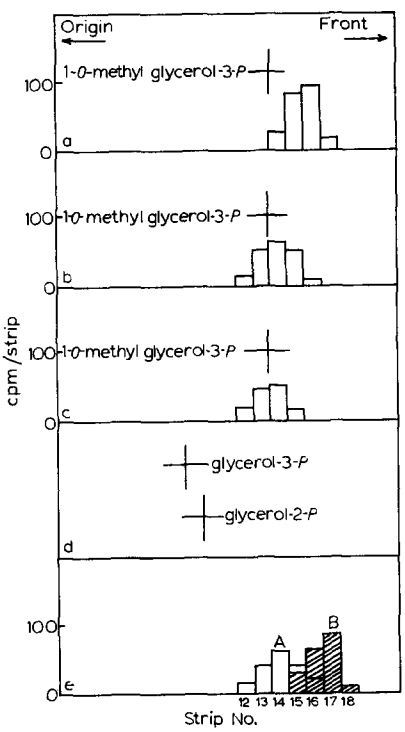

Fig. 2. Thin-layer chromatography of the phosphorylated ^{14}C-labeled compound before and after hydrolysis. Samples a, b and c were mixed with standard 1-*O*-methyl *sn*-glycerol 3-phosphate and pipetted onto Bakerflex cellulose coated strips. Ascending chromatography was carried out with *n*-propanol–conc. NH_4OH–water (6:3:1, by vol.) for 3 h the 1-*O*-methyl *sn*-glycerol 3-phosphate spot was visualized with sulfosalicylic acid spray for phosphates[8]. The chromatograms were then cut into 0.25-inch strips, placed in BBOT–toluene scintillation fluid and counted 10 or 20 min. The counting profile is shown on the edge of the chromatogram tracing. No radioactivity was found on either side of the areas indicated by the profiles. The following samples were analyzed: (a) Purified product as isolated by 0.02 M HCl treatment and eluted from DEAE-Sephadex column. (b) Purified product after alkaline hydrolysis, 1 M KOH at 23 °C for 20 h[16]. (c) Purified product after acid hydrolysis, 0.1 M HCl at 100 °C for 1 h. (d) *sn*-glycerol 3-phosphate and glycerol 2-phosphate. (e) The phosphorylated intermediate released from the protein after isolating the protein-bound product by Sephadex column chromatography. Release was carried out in 0.02 M HCl at room temperature for 30 min. The labeled compound thus obtained was examined before (B) and after (A) alkaline hydrolysis as described in b.

3-phosphate and chromatographed as before. The product of alkaline hydrolysis (Fig. 2b) travels about 5% faster than the 1-*O*-methyl *sn*-glycerol 3-phosphate.

A third portion of the anionic labeled product (300 dpm) was hydrolyzed in 0.1 M HCl 1 h at 100 °C. It was then mixed with the standard and chromatographed as before (Fig. 2c). This time the center of the radioactivity profile coincided with the center of the 1-*O*-methyl *sn*-glycerol 3-phosphate spot.

Thin-layer chromatography of the anionic product after acid hydrolysis using the solvent system butanol–ethanol–water (4:1:5, by vol., upper layer) showed it to coincide with 1-*O*-methyl *sn*-glycerol 3-phosphate.

These results suggest that the product isolated from the 0.02 M $HClO_4$ deproteinization mixture is *X*-1-*O*-methyl glycerol 2:3-cyclic phosphate. The expected product of alkaline hydrolysis of such a cyclic phosphodiester would be 1-*O*-methyl glycerol 2-phosphate[15] while the expected acid hydrolysis product would be 1-*O*-methyl glycerol 3-phosphate[16]. In the chromatographic system *n*-propanol–conc. NH_4OH–water (6:3:1, by vol.) glycerol 2-phosphate travels faster than glycerol 3-phosphate (Fig. 2d) so one would expect 1-*O*-methyl glycerol 2-phosphate to travel faster than 1-*O*-methyl glycerol 3-phosphate, applying the rule of group partition energies[17]. Thus 0.02 M $HClO_4$ treatment of the transmethylation reaction mixtures releases a phosphorylated product which appears to be *X*-1-*O*-methyl glycerol 2:3-cyclic phosphate. However, this would appear to be metabolically a dead end product as it is unlikely to be easily dephosphorylated to the known final product, *rac*-1-*O*-methyl glycerol. The cyclic phosphate is thus thought to be an artifact of the isolation procedure.

Isolation and characterization of the protein-bound labeled product

Our studies into the nature of the anionic labeled product suggest that this product may have been protein-bound. This was demonstrated by passing a transmethylation mixture containing $11.6 \cdot 10^5$ dpm of ^{14}C-labeled *S*-adenosyl methionine (0.3 μmole, 2 mg protein) through a 2 cm × 65 cm column of Sephadex G-10. The ultraviolet-absorbing protein fraction which corresponded to the radioactive fraction was separated from the substrate, recovered and treated with 0.02 M HCl at room temperature for 30 min to release the anionic product. 7000 dpm of anionic material (1.8 nmoles) were obtained. Thin-layer chromatography (Fig. 2e) shows the material to give a single radioactive peak (B) travelling 15% ahead of 1-*O*-methyl *sn*-glycerol 3-phosphate. After alkaline hydrolysis in 1 M KOH for 20 h at 23 °C, a single peak (A) traveling 5% faster than 1-*O*-methyl *sn*-glycerol 3-phosphate appears which corresponds to the expected position of *X*-1-*O*-methyl glycerol 2-phosphate.

In a second experiment, a protein-bound labeled product (synthesized as previously described) containing $2.9 \cdot 10^6$ dpm (2 μmoles, 25 mg protein) in 4 ml was dialyzed at 2 °C against 2 l of 0.03 M phosphate buffer (pH 7.0) containing 7 mM $MgCl_2$. The dialysis medium was changed after 24 h. After 48 h of total dialysis time 9700 dpm (40% of anionic labeled material originally formed in the reaction) remained undialysed. A 0.5-vol. aliquot of the crude dialysis mixture was precipitated with 80% saturated neutral $(NH_4)_2SO_4$ at 0 °C and the residue was washed twice with 80% saturated neutral $(NH_4)_2SO_4$. After this treatment the yield of the counts from the residue was 68%. Another 0.125-vol. aliquot of the dialysis mixture was passed over a 2.9 cm × 62 cm Sephadex G-100 column. Most of the counts were eluted in a broad

peak corresponding to the ultraviolet absorbing protein fraction emerging in the first quarter of the bed volume following the void volume. Thus the protein fraction was directly associated with the radioactivity.

These results indicate that the anionic product which appears to be 1-*O*-methyl *sn*-glycerol 3-phosphate is bound to protein by a very labile covalent linkage. Such a linkage could be associated, for instance, with the imidazole of a histidine residue. The cyclic phosphate product that is formed upon the 0.02 M H^+ treatment could arise from a proton-initiated elimination at the enzyme site. The formation of nucleotide 2′:3′-cyclic phosphates from 3′–5′ oligonucleotide linkages or benzyl 3′-phosphate are examples of this kind of process[18].

DISCUSSION

Previously we reported that *sn*-glycerol 3-phosphate can bind to a protein and can serve as the acceptor of the methyl group from *S*-adenosyl methionine[1]. With the discovery of a protein-bound 1-*O*-methyl *sn*-glycerol 3-phosphate, we can now describe the sequence of reactions leading to the synthesis of 1-*O*-methyl *sn*-glycerol to be: (1) the binding of *sn*-glycerol 3-phosphate to the enzyme, (2) the transfer of the methyl group from *S*-adenosyl methionine to the bound *sn*-glycerol 3-phosphate to form the protein-bound 1-*O*-methyl *sn*-glycerol 3-phosphate and (3) the release of the product, 1-*O*-methyl *sn*-glycerol from the enzyme.

$$\text{Glycerol-3-}P + \underset{P}{\text{Enz}} \rightarrow \text{glycerol-3-}P\text{-Enz-}P \qquad (1)$$

$$S\text{-Adenosyl methionine} + \text{glycerol-3-}P\text{-Enz-}P \rightarrow$$
$$\text{1-}O\text{-methyl glycerol-3-}P\text{-Enz-}P + S\text{-adenosyl homocysteine} \qquad (2)$$

$$\text{1-}O\text{-Methyl glycerol-3-}P\text{-Enz-}P \rightarrow \text{1-}O\text{-methyl glycerol-3-}P\text{-Enz} + P \qquad (3)$$

$$\text{1-}O\text{-Methyl glycerol-3-}P\text{-Enz} \rightarrow \text{1-}O\text{-methyl glycerol} + \underset{P}{\text{Enz}} \qquad (4)$$

The initial presence of the phosphate with its two binding positions at the site makes it possible to order events in such a manner that glycerol is not released prior to methylation (*sn*-glycerol 3-phosphate stays bound). On the other hand, 1-*O*-methyl *sn*-glycerol is released after methylation and the old phosphate group is transferred out. The release or exchangeability of the old phosphate could be dependent on the presence of the methyl group, while the glyceryl moiety stays bound until there is an open binding position to exchange with the phosphodiester bond. In the course of the cycle the enzyme gives up its original phosphate which is replaced by the phosphate from the entering *sn*-glycerol 3-phosphate.

The presence of a phosphodiester intermediate suggested that there might be a requirement for a high-energy compound such as a nucleoside triphosphate associated with the transmethylation reaction. However, experimental work on transmethylase preparations from which endogenous nucleotides had been removed revealed no such requirement (F. A. Lornitzo, unpublished data). This finding is consistent with the above reaction mechanism in which an already preexisting phosphodiester linkage is conserved throughout the cycle.

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