Affinity Purification of Mycobacterial Polymethyl Polysaccharides and a Study of Polysaccharide–Lipid Interactions by ^1H NMR†

Ole Hindsgaul‡ and Clinton E. Ballou*

ABSTRACT: Mycobacterial polymethyl polysaccharides, which bind long-chain fatty acids tightly [Ballou, C. E. (1981) *Pure Appl. Chem. 53*, 107–112], have been purified on a preparative scale by use of an affinity column packing consisting of (palmitoylamino)alkylsilyl silicate. The relatively large amount of material obtained in this way has allowed a study of the polysaccharide–lipid interactions at millimolar concentrations. The anomeric protons for all of the α1→4-linked hexose units in the mycobacterial methylglucose polysaccharide occur in an envelope centered at δ 5.40, and, on titration with hexadecyltrimethylammonium bromide, the majority of these resonances move upfield to about δ 5.15. This shift is consistent with a change in the polysaccharide from a less ordered chain to one that has a significant proportion of helical conformation, and it is probable that the alkyl chain is included in the coiled portion of the polysaccharide in a manner analogous to the interaction of methylmannose polysaccharide with palmitic acid [Yabusaki, K. K., Cohen, R. E., & Ballou, C. E. (1979) *J. Biol. Chem. 254*, 7282–7286]. The native methylglucose lipopolysaccharide, which contains several short-chain acyl groups as well as an esterified octanoyl group, has an anomeric proton nuclear magnetic resonance spectrum similar to that of the methylglucose polysaccharide–hexadecyltrimethylammonium bromide complex. This suggests that the acylation stabilizes the polysaccharide chain in the same conformation it assumes when complexed to a long-chain lipid. Thus, acylation of the methylglucose polysaccharide could have an important role in regulating its shape and lipid-binding properties.

Mycobacteria synthesize two methylated polysaccharides, one composed of 3-*O*-methylmannose and the other of 6-*O*-methylglucose (Ballou, 1981; Yamada & Ballou, 1979; Forsberg et al., 1982). These substances are found in the cytoplasm of the cell where they appear to regulate fatty acid metabolism (Bloch, 1977) as a consequence of their ability to form tight complexes with long-chain acyl coenzyme A derivatives (Machida & Bloch, 1973; Yabusaki & Ballou, 1978; Yabusaki et al., 1979). The 6-*O*-methylglucose polysaccharide is also acylated with acetyl, propionyl, isobutyryl, succinyl, and octanoyl groups (Keller & Ballou, 1968), but the role of acylation is unclear because the deacylated polysaccharide is able to form equally good complexes with long-chain fatty acids. Structures of the polymethyl polysaccharides (PMPS)[1] are shown in Figure 1.

We have previously studied complex formation between lipids and the methylmannose polysaccharide (MMP) and the methylglucose polysaccharide (MGP) by fluorometric titration with parinaric acid (Yabusaki & Ballou, 1978), which suggested that both polysaccharides bound this lipid with the same affinity (K_d about 0.4 μM). From NMR studies, it has also been demonstrated that MMP undergoes a dramatic change in conformation when it forms a complex with palmitic acid (Yabusaki et al., 1979; Maggio, 1980), and the results give strong support to a model in which the fatty acid is included in the nonpolar cavity of the coiled polysaccharide chain.

In the present study, we provide evidence from NMR data that MGP undergoes a similar change in conformation when a complex is formed with a long alkyl chain. We were surprised to observe, however, that the native methylglucose lipopolysaccharide (MGLP) gave a NMR spectrum in the absence of added long-chain fatty acid that suggested it has a coiled conformation even in the free state. We conclude that the acylation of MGP to form MGLP stabilizes a more ordered conformation, which may result from the folding of the polysaccharide chain around the octanoyl chain that is known to be esterified to the glyceric acid portion of the molecule (Smith & Ballou, 1973). This acylation of MGP may alter its fatty acid binding specificity and serve to fine tune the regulatory activity this substance has in the cell.

Experimental Procedures

Materials

Aminoalkylsilyl silicate (lot AE2-142, 18 μmol of $-NH_2$/g) was a gift from Chembiomed Ltd., Edmonton, Alberta, Canada; *p*-nitrophenol and *N,N'*-dicyclohexylcarbodiimide were from Aldrich Chemical Co.; CH_2Cl_2 and CH_3CN were analytical-grade reagents from MC/B; cyclodextrins and hexadecyltrimethylammonium bromide (HTMA) were from Sigma; fatty acids were from Nutritional Biochemicals Corp. All other reagents were analytical grade and were purified by distillation or recrystallization when appropriate. *Mycobacterium smegmatis* ATCC 356 cells were grown and harvested according to Gray & Ballou (1975), and the wet cell paste was kept frozen until needed.

Methods

(Fatty acid)(acylamino)alkylsilyl silicate derivatives were formed by the reaction of the aminoalkylsilyl silicate with fatty acid *p*-nitrophenyl esters that were prepared by coupling the free fatty acid to *p*-nitrophenol in the presence of dicyclohexylcarbodiimide. The extent of substitution of the support

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‡ Present address: Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada.

[1] Abbreviations: PMPS, polymethyl polysaccharide(s); MMP, methylmannose polysaccharide; MGP, methylglucose polysaccharide; AGMGP, amylase/glucoamylase-digested MGP; MGLP, methylglucose lipopolysaccharide; HTMA, hexadecyltrimethylammonium bromide; GL, glycolipid; CoA, coenzyme A.

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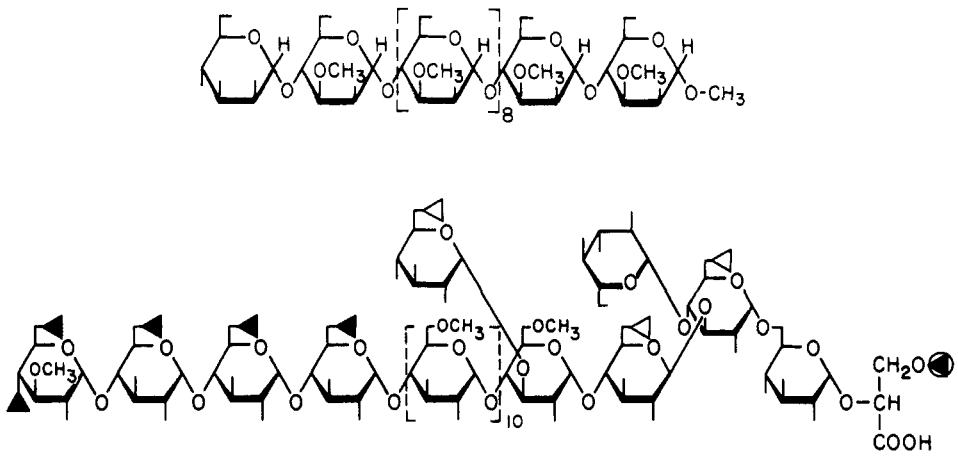

FIGURE 1: Structures of the polymethyl polysaccharides. The top figure is MMP-III with 8 of the 3-*O*-methylmannose units in brackets, and the bottom figure is MGLP with 10 of the 6-*O*-methylglucose units in brackets, and with the positions of substitution by neutral (▲) and acidic (△) acyl groups indicated. The octanoyl group is specified by a solid triangle enclosed in an open circle. MGP is deacylated MGLP, and AGMGP is formed by enzymic removal of the four hexoses at the nonreducing end of MGP.

was calculated from the amount of free fatty acid and ester recovered from the reaction mixture.

The adsorbent was packed into a 4-cm glass column to a height of 45 cm for preparative chromatography, whereas smaller columns were used for analytical studies. Cell extracts were applied to the column in water and, after unbound material was eluted with water, the adsorbed components were eluted with 50% ethanol.

Sugar compositions of eluted fractions were determined by gas chromatography of the reduced and acetylated monosaccharides (Lindberg, 1972) obtained by hydrolysis in 2 N trifluoroacetic acid at 120 °C for 2 h. A 3% OV-210 column (0.3 × 120 cm) was used at 200 °C with a He flow rate of 40 mL/min, under which conditions 6-*O*-methylglucose had a retention time of 4.4 min, 3-*O*-methylmannose 5.9 min, mannose 6.4 min, and glucose 7.5 min.

Proton NMR spectra were determined in 100% D_2O on a 250-MHz spectrometer equipped with a superconducting magnet and a Nicolet 1180 computer operated in the Fourier-transform mode (at the Chemistry Department, University of California, Berkeley) or at 360 MHz (at the Department of Chemistry, University of Alberta, Edmonton, Canada).

Results

Preparation of Affinity Adsorbent. The palmitoyl derivative of aminoethyl-Bio-Gel P-150 has been used previously for the affinity adsorption of methylmannose polysaccharide from *Streptomyces griseus* (Kari & Gray, 1979). In the present study, we have used fatty acid derivatives of aminoalkylsilyl silicate. To allow careful control of the extent of acylation of the support, we have reacted it with fatty acid *p*-nitrophenyl esters (λ_{max} = 270 nm) and followed the reaction by the disappearance of ester or by the appearance of free *p*-nitrophenol (λ_{max} = 307 nm).

p-Nitrophenyl palmitate was prepared by dissolving 334 mg of *p*-nitrophenol (2.4 mmol) and 512 mg of palmitic acid (2.0 mmol) in 8 mL of ethyl acetate and 2 mL of CH_2Cl_2. To this solution was added 494 mg of dicyclohexylcarbodiimide (2.4 mmol) dissolved in 2 mL of ethyl acetate, and the mixture was stirred for 3 h at 23 °C during which a precipitate of dicyclohexylurea formed. Acetic acid (100 μL) was added, and the stirring was continued for 15 min, after which the mixture was filtered and the filtrate was evaporated to dryness under vacuum. The pale yellow residue was dissolved in 30 mL of hot 95% ethanol, from which crystals of *p*-nitrophenyl palmitate separated on cooling. The crystals were collected by filtration, washed 3 times with 15-mL portions of 95% ethanol, and then were dried under vacuum. The yield was 350 mg (46% of theory).

In a 2-L round-bottomed flask, 100 g of aminoalkylsilyl silicate was suspended in 300 mL of CH_3CN/CH_2Cl_2 (4:1 v/v), and 191 mg (0.5 mmol) of *p*-nitrophenyl palmitate was added as a solution in the same solvent. The flask was then attached to a motor and rotated slowly for 72 h at 23–26 °C (room temperature) to minimize breakage of the beads. To follow the reaction, 100-μL samples were removed, diluted with CH_2Cl_2, filtered to remove solids, and evaporated to dryness. The residue was dissolved in CH_3CN (1.0 mL), and the absorbance was determined at 270 nm. The half-time for the reaction under these conditions was about 5 h.

At completion of the reaction, the solid was recovered by vacuum filtration and was washed twice with 300-mL portions of CH_2Cl_2. Evaporation of the combined filtrates yielded 64 mg of *p*-nitrophenol and 6 mg of *p*-nitrophenyl palmitate. The solid adsorbent was washed with methanol and then stirred for 2 days with 10% acetic anhydride in methanol to acetylate all unreacted amino groups. Finally, the adsorbent was washed sequentially with methanol, water, saturated sodium bicarbonate solution, water, methanol, and CH_2Cl_2, and then it was dried in air. Myristic and stearic acids were coupled in a similar fashion.

Properties of the Acylated Adsorbent. The palmitoyl silicate adsorbent (which contained about 5 μmol of palmitic acid/g) was very hydrophobic and floated in water, but it could be wet by 50% ethanol and, in this solvent, the adsorbent was conveniently packed into a chromatography column, after which it was washed thoroughly with water to remove the ethanol. When an aqueous solution of purified methylmannose polysaccharide or methylglucose lipopolysaccharide was added and the column was washed with water, the polysaccharide was bound, but it could be eluted with 50% ethanol. The capacity per gram of the adsorbent was 1.13 mg of MMP and 1.26 mg of MGLP, which amounts to about 0.5 μmol of either polysaccharide or about 10% of the theoretical capacity. Thus, most of the fatty acid on the adsorbent is not accessible to the polysaccharides.

The palmitoyl silicate adsorbent failed to bind amylo-oligosaccharides, such as an α1→4-glucan with 18 hexose units, but it did bind with variable efficiencies the cyclodextrins (Figure 2). Under the test conditions, all were somewhat retarded in aqueous solution, and elution with 50% ethanol indicated that α-cyclodextrin was 16% bound, β-cyclodextrin

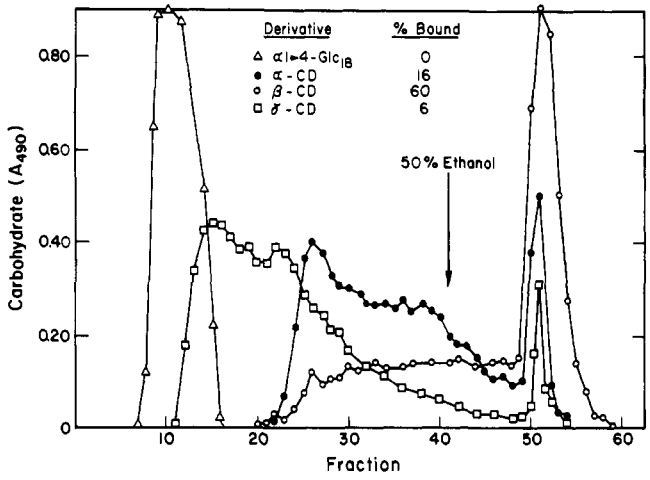

FIGURE 2: Interaction of cyclodextrins with the fatty acid affinity column. Aqueous solutions of the three cyclodextrins and amylooctadecasaccharide were applied individually to the column, which was eluted first with water and then with 50% ethanol as indicated on the figure. The fractions were tested for carbohydrate by the phenol–sulfuric acid reaction. The amylooctadecasaccharide (△) failed to bind, α-cyclodextrin (●) bound weakly, β-cyclodextrin bound more strongly (○), and γ-cyclodextrin (□) appeared to bind very poorly. As expected, the extent of retardation was related to the degree of binding.

was 60% bound, and γ-cyclodextrin was only 6% bound. This binding selectivity probably reflects a discrimination based on the size of the cavity, which determines the ease with which the complex can form, and the stabilities of the resulting complexes, which are determined by a number of factors (Saenger, 1980).

Preliminary Studies on the Affinity Isolation of Polymethyl Polysaccharides. Frozen *M. smegmatis* cells (143 g wet weight) were extracted under reflux for 2 h with a mixture of absolute ethanol (543 mL) and water (90 mL). After it had cooled, the mixture was filtered under vacuum, and the filtrate was evaporated to dryness. The solid residue was partitioned between the two layers of a chloroform/methanol/water (8:4:3) mixture (430 mL). After the mixture had remained at room temperature (23 °C) for 2 days for the emulsion to clear, the upper aqueous layer was removed and decolorized by 8 g of charcoal (Norit A, activated) with heating of the mixture on the steam bath. The charcoal was removed by filtration and washed with 200 mL of 1:1 methanol:water. The combined filtrates were evaporated to a syrup which was dissolved in 10 mL of water, and the solution was loaded on a 55-g palmitoyl silicate affinity column. On elution with water, the bulk of the carbohydrate material passed through, and when the base line for the phenol–sulfuric acid assay returned to 0 (after 15 fractions of 20 mL each), the column was eluted with 50% ethanol. The displaced carbohydrate appeared in fractions 20–25 and amounted to 41.5 mg. The carbohydrate eluted in the first 15 fractions was passed a second time through the same column in water, and on elution with 50% ethanol, very little phenol–sulfuric acid positive material was obtained. The material eluted with 50% ethanol was hydrolyzed to monosaccharides which were analyzed as the polyol acetates by gas chromatography. The major peak was 3-*O*-methylmannose, but 6-*O*-methylglucose was not present. Subsequent studies revealed that the decolorizing charcoal adsorbed all of the methylglucose lipopolysaccharide and a contaminating acylated glucooligosaccharide; if this step was eliminated, the material bound to the affinity column contained these substances as well as the methylmannose polysaccharide.

Large-Scale Isolation of Polymethyl Polysaccharides. Several difficulties were encountered when the isolation was carried out by starting with several kilograms of cells. First, the extraction with 70% ethanol had to be repeated at least 3 times in order to obtain most of the PMPS. When each extract was analyzed, it was found that the first was enriched in MMP whereas the later ones were enriched in MGLP. A second problem was encountered with foaming during evaporation of the water extract, and antifoam added to minimize this difficulty had to be removed later by an ether extraction; otherwise it interfered with the Sephadex G-50 column fractionation. Finally, it was discovered during the isolation that the cell extracts contained a glycolipid that bound to the affinity column along with the PMPS. This reduced the capacity of the column for PMPS and necessitated an additional step for separation of the glycolipid from the PMPS. The structure of this glycolipid is reported elsewhere (Saadat & Ballou, 1983).

Frozen *M. smegmatis* cells (8.9 kg) were extracted batchwise with 45 L of technical-grade acetone for 24 h at room temperature (23 °C) and then were collected by vacuum filtration on glass fiber paper. This solid residue was stirred in a 200-L tank with 70% ethanol (67 L) for 2 h at 65–70 °C. After the mixture was cooled to 0 °C, the solid and liquid phases were separated on a large, electrically driven Sharples centrifuge. A second extract was made in a similar manner and kept separate, leaving 4.0 kg of dry cell residue.

The two ethanol extracts were kept several days at 23 °C during which a waxy solid separated. The clear liquid from each was decanted from the solid and concentrated on a steam-heated cyclone evaporator to about 8 L in the presence of 2-octanol to minimize foaming. A final concentration was done under vacuum on a rotary evaporator at 50 °C, to yield an aqueous slurry that was frozen and lyophilized. The first ethanol extract yielded 292 g of solid material, and the second gave 82 g.

The first ethanol extract (292 g) was partitioned between the two layers of a $CH_2Cl_2/CH_3OH/H_2O$ mixture (8:4:3), 18-L total volume. The emulsion that formed cleared after 4 days at 23 °C, and the upper aqueous layer was evaporated at 50 °C on a rotary evaporator in the presence of a few drops of Dow-Corning SAG-471 antifoam agent. The residue was dissolved in 500 mL of water, filtered through glass wool, and diluted to 1 L with ethanol to give a dark brown solution that was stored as such until needed for the next step. The second ethanol extract was treated in a similar fashion, and the water-soluble residue in 100 mL of water was diluted to 400 mL with ethanol to also give a dark brown solution. These extracts could be decolorized with activated charcoal, but charcoal adsorbed the MGLP so the step was eliminated.

Preliminary studies showed that a column containing 250 g of the affinity adsorbent (4 × 45 cm packed bed) was able to bind all of the PMPS and glycolipid in 50 mL of the 50% ethanol extract prepared above. Before application to the column, which had been packed in 50% ethanol and then washed with water until free of ethanol, the extract was evaporated to dryness and redissolved in 50 mL of water. This solution was applied to the column which was then eluted with water. Fractions of 300 drops (about 20 mL) were collected, with the bulk of the carbohydrate appearing between fractions 22 and 35. After 70 fractions were eluted, the solvent was changed to 50% ethanol, which caused the bound carbohydrate material (amounting to 2.5% of that applied to the column) to be eluted in fractions 92–110. The column was regenerated by washing it with water, and then it was reused for processing

Table I: Compositions of Fractions Isolated by Affinity Chromatography

fraction[a]	sugar composition[d] (molar ratio) Glc	MeGlc	Man	MeMan	total wt (mg)	probable identity
			First Ethanol Extract			
fractions 51–90	5	5	1	8	48	PMPS
fractions 91–130	5	2	1	8	340	GL + PMPS
G-50 void	2.5	1	*b*	*b*	163	GL
G-50 included	2	2.2	1	8.2	151	PMPS
fractions 131–170	2	*b*	*b*	1	26	unknown
			Second Ethanol Extract			
fractions 51–90	7.4	5.8	*b*	1	50	MGLP (85%)
fractions 91–130	3.8	2	1	4	280	PMPS
G-50 void					112	GL
G-50 included					143	PMPS
			Third Ethanol Extract			
fractions 91–130					467	MGLP
G-50 void					224	*b*
G-50 included					196	MGLP
			Calcd Total/10 kg of Cells			
fractions 91–130					10700	GL + PMPS
G-50 void					4593	GL
G-50 included					4510	PMPS[c]

[a] Consisting of 300 drops/fraction from a 4 × 45 cm column of adsorbent. [b] Not analyzed or quantified. [c] If it is assumed that 10 kg of cells has an internal volume of 5 L, this indicates a PMPS concentration of about 0.4 mM, which agrees with previous estimates. [d] Abbreviations: Glc, glucose; MeGlc, methylglucose; Man, mannose; MeMan, methylmannose.

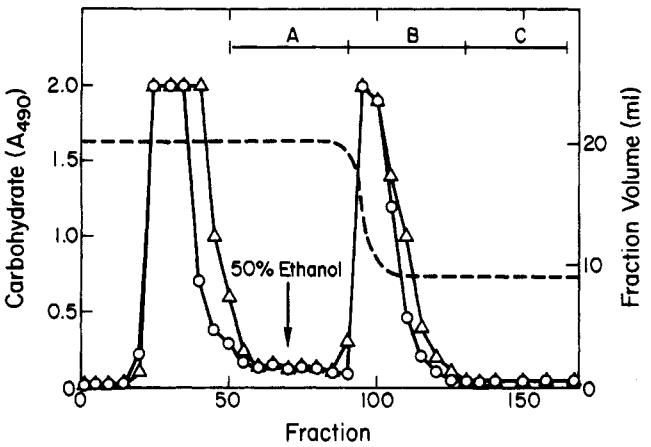

FIGURE 3: Isolation of polymethyl polysaccharides. The elution of unbound and bound carbohydrates is shown along with the fraction volumes, which change due to the effect of ethanol on the drop size. The bound carbohydrate represented 2.5% of that applied to the column, and the column efficiency did not change between the first (○) and tenth (△) isolation.

the remainder of the material. No loss in capacity was apparent on comparing the elution profiles for the first and tenth separations (Figure 3). Note that during the elution with 50% ethanol the drop size changes as the ethanol solution passes from the column so that the fraction size is decreased to about 9 mL.

The column effluent was combined in three pools, fractions 51–90, 91–130, and 131–170. The weight of carbohydrate and the monosaccharide compositions of each are given in Table I, along with the proposed oligomeric compositions. It is apparent that some PMPS is released from the column during the water wash, but the bulk is eluted in fractions 91–130 along with a glycolipid that accounts for the high glucose content of this pool. To separate the glycolipid from the PMPS, the solvent was evaporated to remove the ethanol, the volume was adjusted to 100 mL with water, and the solution was extracted 5 times with 20-mL portions of diethyl ether to remove the antifoam, and then it was lyophilized. A 70-mg portion of the solid was dissolved in 0.1 N acetic acid and fractionated on a Sephadex G-50 column by elution with the same solvent. The void volume carbohydrate (31 mg) contained the glycolipid whereas the included material (fractions 50–65) contained the PMPS. The exact position of elution of the PMPS varied slightly because of the effect of detergent or lipids on the drop size. Analyses of these fractions are given in Table I, and it is seen that the glycolipid contains glucose and 3-*O*-methylglucose while the PMPS fraction contains glucose, 6-*O*-methylglucose, mannose, and 3-*O*-methylmannose. The ratio of the first two sugars in the PMPS fraction is that expected for the methylglucose lipopolysaccharide, and the ratio of the last two is that expected for the methylmannose polysaccharides.

A 50-mL portion of the second ethanol extract of *M. smegmatis* cells was processed in a similar fashion to yield 280 mg of carbohydrate from the affinity column in fractions 91–130, 40% of which was in the PMPS fraction after filtration on Sephadex G-50, and 80% of this was methylglucose lipopolysaccharide.

One kilogram of the ethanol-extracted cells was extracted twice more with boiling 70% ethanol, and 4.3 g of solid material was obtained after the partitioning in CH_2Cl_2/CH_3OH/H_2O. Affinity chromatography of this material gave 470 mg of desorbed glycolipid/PMPS fraction which was separated on the Sephadex G-50 column to yield 195 mg of PMPS that proved to be almost pure methylglucose lipopolysaccharide (Table I).

From the three 70% ethanol extractions, a total of about 10 g of the glycolipid–PMPS mixture was obtained from 9 kg of cells. As the separation on Sephadex G-50 shows, this is composed of equal amounts of the two components.

Fractionation of Polymethyl Polysaccharides. When applied to a DEAE-Sephadex column, the neutral methylmannose polysaccharides pass through (Gray & Ballou, 1975), and the acidic methylglucose lipopolysaccharide isomers bind and may be eluted with a salt gradient (Keller & Ballou, 1968). The individual lipopolysaccharide fractions are pooled and lyophilized to remove the salt, whereas the methylmannose fraction (methylmannose to mannose ratio = 12.7) is resolved by high-pressure liquid chromatography to obtain the indi-

Table II: Low-Field Anomeric Proton Chemical Shifts and Integrations of 6-*O*-Methylglucose Polysaccharide Derivatives

sample	chemical shifts (δ) and no. of protons[b]				
MGP	5.47 (1)	5.40 (15)			
AGMGP	5.48 (1)	5.40 (10)	5.35 (1)		
MGP-HTMA	5.46 (1)	5.41 (1)	5.35 (1)	5.25 (1)	5.15 (11)
			5.32 (1)		
AGMGP-HTMA	5.41 (1)		5.34 (1)	5.25 (1)	5.19 (8)
			5.32 (1)		
MGP-palmitoyl-CoA		5.38 (1)	5.32 (1)	5.2 (2)	5.1 (12)
MGLP-I		5.38 (1)	5.25 (5)	5.16 (9)	
MGLP-II			5.29 (6)	5.18 (10)	
MGLP-III				5.18 (6)	5.12 (10)
MGLP-IV				5.18 (7)	5.11 (9)
MGLP-II-HTMA					5.12 (12)[a]

[a] These are centered at δ 5.12 but are distributed in a broad envelope from δ 5.20 upfield to δ 5.05, and the remaining four protons at lower field were so broadened as to be lost in the base line. [b] In parentheses.

vidual homologues (Yamada & Ballou, 1979). A portion of the methylglucose lipopolysaccharide mixed isomers was deacylated and isolated by gel filtration on a Bio-Gel P-4 column. The ratios of 6-*O*-methylglucose:glucose:3-*O*-methylglucose were 11.9:7.3:1, whereas the expected values are 11:8:1 (Forsberg et al., 1982). The PMPS fraction (49 mg) from the first ethanol extract gave 32 mg of MMP and 14 mg of MGLP, while the second extract gave a much higher proportion of MGLP, and the last extract gave solely MGLP.

Interaction of Polymethyl Polysaccharides with Hexadecyltrimethylammonium Bromide. Complex formation between MMP or MGP and palmitate or palmitoyl coenzyme A was previously demonstrated by fluorometry (Yabusaki & Ballou, 1978) and by NMR (Yabusaki et al., 1979). Because the solubilities of these lipids are low [the critical micellar concentrations are between 5 and 40 μM (Zahler et al., 1968; Powell et al., 1981)], it was not possible to demonstrate interactions with polysaccharides that have much lower affinities than the PMPS. In the present study, we have used hexadecyltrimethylammonium bromide as the lipid, and its high solubility (about 10% in water) has allowed us to measure complex formation in the millimolar range of reactants by NMR.

The chemical shift of the anomeric protons in a linear, presumably randomly coiled, α1→4-glucooligosaccharide is about δ 5.38, whereas in the conformationally fixed α-cyclodextrin the signal appears at δ 5.04 (Yabusaki et al., 1979). This difference can be used as an intrinsic probe of the change from a large to a small dihedral angle in the glycosidic bond between the sugars in the chain (Casu et al., 1977). The bulk of the anomeric protons in MMP appear at δ 5.22, and they move upfield to δ 5.00 upon titration with palmitate. Because this shift parallels the difference between the linear and cyclic dextrins, it has been interpreted as a change in the conformation of the MMP from a loose to random coil to a tightly coiled chain that is stabilized by an included palmitate molecule (Yabusaki et al., 1979).

In Figure 4, we have recorded partial NMR spectra for the anomeric proton regions of MMP, MGP, and AGMGP at 1 mM concentrations in the presence and absence of hexadecyltrimethylammonium bromide also at 1 mM concentration. For each oligosaccharide, the bulk of the anomeric protons appear in an envelope, and most of them are shifted upfield on addition of the lipid, which suggests that characteristic inclusion complexes are formed. This result was expected for MMP and MGP, but it is a novel observation that AGMGP also shows chemical shifts in the presence of hexadecyltrimethylammonium bromide expected for a polysaccharide–lipid inclusion complex.

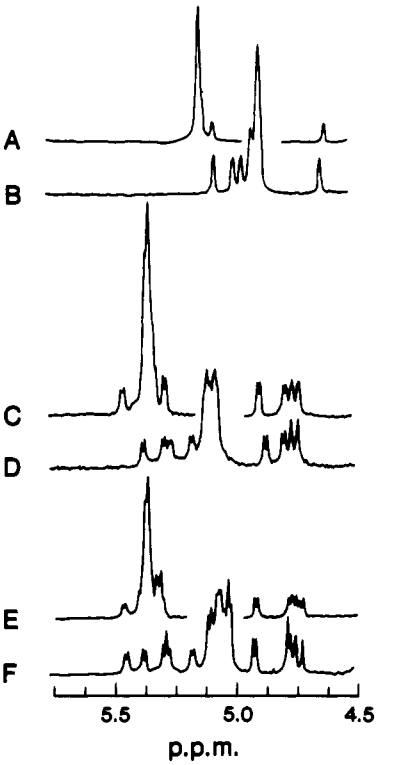

FIGURE 4: Demonstration of polymethyl polysaccharide–lipid interaction by NMR. (A, C, and E) 360-MHz anomeric proton spectra of MMP-III, AGMGP, and MGP, respectively. (B, D, and F) Spectra of equimolar mixtures of hexadecyltrimethylammonium bromide with MMP-III, AGMGP, and MGP, respectively. The concentrations were about 1 mM polysaccharide and lipid, and the spectra were determined at 40 °C.

From analysis of each of the three experiments in Figure 4, it is seen (Table II) that MMP-III has 10 protons in the envelope centered at δ 5.22 and 8 of these move upfield to δ 5.02 in the presence of lipid whereas 2 other protons are only slightly affected. MGP has 15 anomeric protons centered at δ 5.40; 11 of these shift to δ 5.15, and 2 others are moved slightly upfield. Finally, AGMGP has 10 protons at δ 5.40, and 8 of these are shifted to δ 5.19. Again, two protons become slightly more shielded in the complex. The general conclusion is that in the lipid complexes with MMP and AGMGP, 8 hexoses interact strongly with the lipid and 2 others interact weakly, whereas with MGP there are 11 hexoses that interact strongly and 2 weakly.

Similar studies were done with an α1→4-glucooligosaccharide with 13 hexose units (data not shown). In the anomeric proton region, the major envelope appeared as a

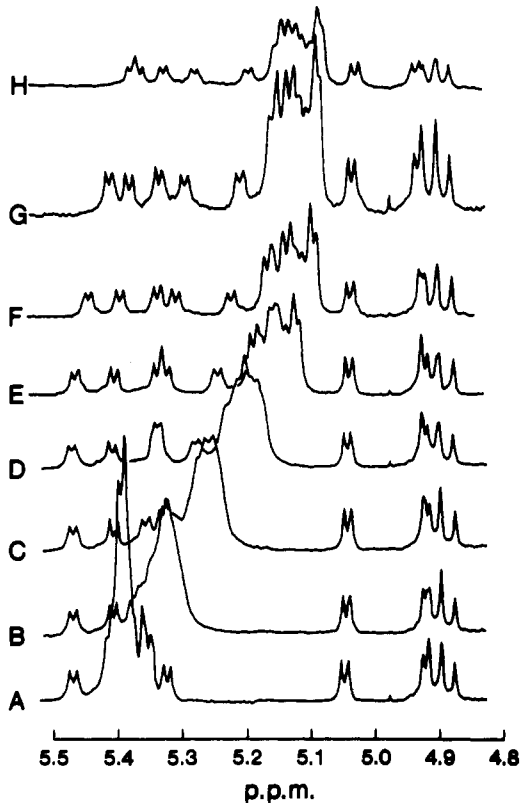

FIGURE 5: Titration of MGP with hexadecyltrimethylammonium bromide. (A) The anomeric proton spectrum for 1 mM MGP; (B–H) the spectra for 1 mM MGP with 0.28, 0.56, 0.72, 0.96, 1.20, 1.51, and 1.69 mM lipid, respectively. A plot of $\Delta\delta$ for the major signal at 5.4 ppm against lipid concentration gives a straight line with the break point occurring at a lipid concentration of 1.09 mM.

doublet at δ 5.38, and in presence of an equimolar amount of hexadecyltrimethylammonium bromide, both at 1 mM concentration, the envelope was shifted to δ 5.22. Although the base of the signal at δ 5.22 was broadened, it appeared that all of the anomeric protons in the oligosaccharide were strongly affected, in contrast to the observations with methylated oligosaccharides.

The spectral changes observed on titrating 1 mM MGP with hexadecyltrimethylammonium bromide are shown in Figure 5. The shift in the envelope of signals at 5.40 ppm is dramatic, whereas the signals at 4.8–5.1 ppm are not affected even at the highest lipid concentration. Moreover, only when the lipid to polysaccharide ratio exceeds 1 does the signal at 5.48 ppm begin to move. This signal has been assigned to the side-chain glucose in α1→3 linkage, and the result suggests that this hexose is perturbed only after more than one lipid molecule is available to interact with the polysaccharide.

Competition between Oligosaccharides for Lipid. Although it is known that MMP and MGP bind parinaric acid with equal affinities, AGMGP shows no evidence of interaction at 1 μM concentration of reactants (Yabusaki & Ballou, 1978). Therefore, in the interaction with hexadecyltrimethylammonium bromide at 1 mM concentrations, MMP and MGP should compete equally with each other whereas AGMGP should be a poor competitor with both oligosaccharides. The results in Figure 6 demonstrate that these expectations are generally met, although AGMGP appears to compete much better than predicted from the measurements made at low concentrations. In a mixture of MMP, MGP, and hexadecyltrimethylammonium bromide, 1 mM each, the main anomeric proton envelope of MMP is shifted upfield 35% of that found for the fully complexed molecule whereas the MGP

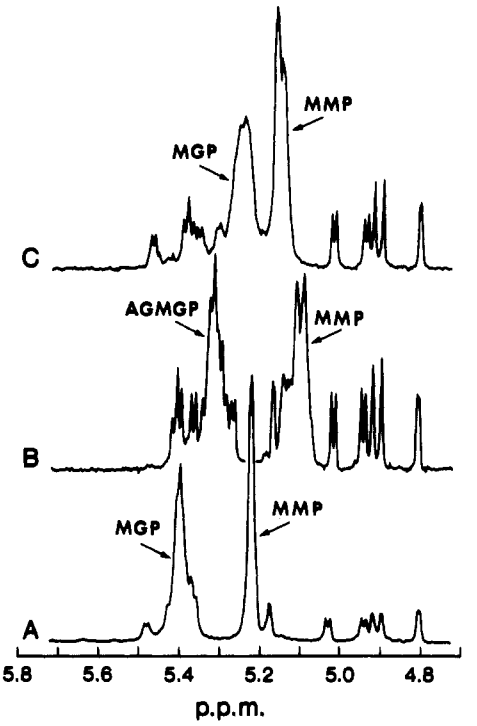

FIGURE 6: Competition between polymethyl polysaccharides for lipid. (A) 360-MHz anomeric proton spectrum of a mixture of MMP and MGP (1 mM each). (B and C) Spectra of MMP–AGMGP and MMP–MGP solutions after addition of HTMA to 1 mM concentration. The assignments of the major proton signals are indicated on each spectrum.

envelope is shifted 60% of the maximum value. For a mixture of MMP and AGMGP, the envelope of the MMP protons is shifted 60% of the maximum and that of the AGMGP only 43%.

Methylglucose Lipopolysaccharide (MGLP) Interaction with Lipid. Although MGP interacts strongly with long-chain fatty acids, the polysaccharide is present in the cell in a highly acylated form that contains acetate (3 mol), propionate, isobutyrate, and octanoate (1 mol of each), and 0–3 mol of succinate (Keller & Ballou, 1968). The anomeric proton NMR signals of MGLP isomers that differ in succinate content are listed in Table II. The surprising observation is that all of the MGLP molecules, in the absence of added lipid, show anomeric proton chemical shifts characteristic of the coiled conformation. Moreover, the upfield shifts increase with the content of succinate, MGLP-I having none and MGLP-IV having 3 mol of this acid. One interpretation of these results is that the polysaccharide chain forms an intramolecular inclusion conformer with the octanoyl group, acylated to the glyceric acid unit, and that this conformer is stabilized by the succinate groups through the chelation of cations. On addition of hexadecyltrimethylammonium bromide to MGLP-II, there is evidence of complex formation in the slight, but clear, upfield shift of the main envelope of anomeric protons (Table II). This suggests that the longer chain of the added lipid may be able to disrupt the presumed intramolecular complex in order to form an intermolecular inclusion compound similar to that formed with MGP.

Discussion

This research has extended our studies on the mycobacterial polymethyl polysaccharides in two important ways. First, we have developed a general method of isolation that allows preparation of gram quantities of the substances, and second,

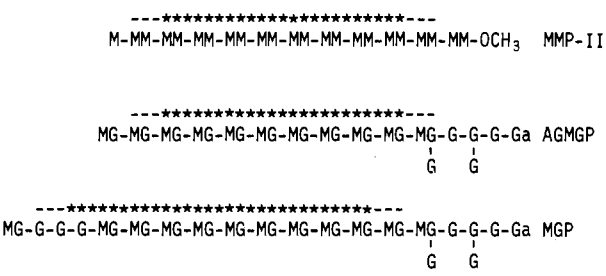

FIGURE 7: Schematic representation of postulated fatty acid binding sites on polymethyl polysaccharides. The asterisks above each structure represent sites of strong interaction, and the dashed lines are sites of weak interaction. MMP-III is the methylmannose polysaccharide with 12 hexose units, MGP is methylglucose polysaccharide, and AGMGP is amylase/glucoamylase-digested MGP.

we have enlarged the range of polysaccharide–lipid interactions that can be studied by employing a water-soluble fatty acid analogue, hexadecyltrimethylammonium bromide (HTMA). At this time, a question of interest is how many and which sugars are directly involved in the polysaccharide–lipid complexes? We know that at low (1 μM) concentrations, MGP interacts with parinaric acid with a dissociation constant of about 0.4 μM, whereas there is no evidence of complex formation by AGMGP (Yabusaki & Ballou, 1978). At 1 mM concentrations, however, both MGP and AGMGP form complexes with HTMA as evidenced by the chemical shift experienced by certain of the anomeric protons. AGMGP has a chain of 11 6-*O*-methylglucoses in α1→4 linkage, and the anomeric protons of all of these occur in an envelope centered at δ 5.40. In the complex with HTMA, eight of these anomeric protons are shifted to δ 5.19, and three are left behind at δ 5.25, 5.32, and 5.34. Because the 6-*O*-methylglucose nearest the glyceric acid end of the chain is substituted at position 3 by an α-glucose unit (δ 5.48), this methylhexose may be prevented from interacting strongly with the lipid, and its anomeric proton may be one that is not strongly shifted. We note, however, that the anomeric proton of the side-chain glucose unit is slightly shifted in the complex from δ 5.48 to δ 5.41, so this portion of the polymer chain is somewhat affected by the lipid. An important conclusion is that the AGMGP–lipid complex involves only 8 of the 11 methylglucose units (Figure 7) and that this in part accounts for the weak interaction.

In contrast to this observation, MGP has 15 hexoses in α1→4 linkage, and 15 anomeric protons appear in an envelope at δ 5.40. In the complex with HTMA, 11 of these are shifted upfield to δ 5.15, and 4 remain behind at δ 5.25, 5.32, 5.35, and 5.41. It is probable that three of these hexose units correspond in their environments to the three anomeric protons that are only weakly shifted in AGMGP, and the fourth may be the 3-*O*-methylglucose unit that is absent in AGMGP but present at the nonreducing terminus of MGP. The signal at δ 5.47 for the side-chain glucose in α1→3 linkage is only slightly affected in the MGP–lipid complex. The results summarized here suggest that 11 of the hexoses in MGP interact strongly with HTMA in the complex (Figure 7) and that this accounts for its greater complexing ability.

Analysis of the MMP–lipid complex provides a suggestion as to which of the α1→4-linked hexoses in MGP and AGMGP are only weakly involved in the lipid complex. Because MMP is all α1→4 linked, we can distinguish the anomeric protons of the sugar unit at each end of the chain from those in the middle. MMP-III has 12 hexose units, 10 of the 3-*O*-methylmannose anomeric protons appearing in an envelope at δ 5.22, the reducing end 3-*O*-methylmannose anomeric proton resonating at δ 4.80 and the nonreducing end mannose anomeric proton resonating at δ 5.18. MMP-III will form a complex with palmitic acid at 1 μM concentration, and 8 of the 10 anomeric protons at δ 5.22 are shifted upfield to δ 5.00. The anomeric protons for the reducing end 3-*O*-methylmannose and the nonreducing end mannose are unaffected, whereas two of the protons at δ 5.22 are only slightly shifted to δ 5.05 and 5.11. We have intepreted this to mean that eight of the hexoses in the middle of the chain interact strongly with the lipid; the penultimate hexoses at each end interact weakly, and the two termini do not interact at all (Figure 7). The proton NMR spectrum of MMP-III complexed with HTMA at 1 mM concentrations is almost identical with that just described for MMP-III–palmitate, and the result is very similar to that for AGMGP–HTMA. This suggests that the hexoses in AGMGP that interact most strongly with the lipid are those eight that follow the branched 6-*O*-methylglucose unit. This would leave the 6-*O*-methylglucose unit at the nonreducing end unaffected (the signals at δ 5.33 in the free and complexed AGMGP), and it would cause weak shifts in the penultimate and the branch-point 6-*O*-methylglucoses.

This analogy can now be extended to the MGP–HTMA complex to accommodate the fact that it possesses a longer α1→4-linked hexose chain and the lipid interacts strongly with three more hexose units than in either MMP or AGMGP. Because there is little effect on the signal at δ 5.47 for the α1→3-linked glucose side chain, we conclude that the lipid in the complex is shifted toward the nonreducing end and interacts strongly with two glucoses and nine 6-*O*-methylglucoses. This would leave one glucose near the nonreducing end and one 6-*O*-methylglucose near the other end of the chain in positions to interact weakly with the lipid. In this model, the 3-*O*-methylglucose at the nonreducing end and the branch-point 6-*O*-methylglucose would remain unaffected in the complex.

Our results reveal the anomalous facts that although both MMP-III and AGMGP form a complex with HTMA at 1 mM concentrations involving a strong interaction with eight hexoses, AGMGP does not form a demonstrable complex with parinaric acid at 1 μM concentration. In contrast, MGP interacts with lipid through 11 hexose units and gives a K_d of 0.4 μM with parinaric acid. Whereas we earlier (Yabusaki & Ballou, 1978) attributed this difference in binding ability between MGP and AGMGP to the difference in the length of the α1→4-linked section of the molecules, it now appears that some other structural feature must also be important. The explanation probably lies in the diverse linkages and branching that are found near the glyceric acid end of the 6-*O*-methylglucose polysaccharides. In particular, it seems likely that the branched 6-*O*-methylglucose unit is unable to participate in the polysaccharide–lipid interacting system, so that the active portion of AGMGP is only 10 hexoses long. It is known that although MMP-III (12 hexoses) interacts well with lipids, the homologue MMP-IV (11 hexoses) interacts very poorly (Yabusaki & Ballou, 1978). Thus, there is a sharp discontinuity in the chain-length dependence of the polysaccharides with regard to their ability to bind fatty acids, and any break in the structural uniformity of the molecule such as branching could affect this property.

Although MMP and AGMGP form similar complexes with HTMA, MMP should compete effectively against AGMGP when in a mixture with a limited amount of HTMA. In a solution 1 mM each in MMP-III, AGMGP, and HTMA, the anomeric proton chemical shifts of MMP are 60% of the maximum whereas the chemical shifts of AGMGP are only

43% of the maximum. This is not as great a difference as one would expect in view of their relative abilities to bind parinaric acid.

Palmitoyl-CoA is an inhibitor of many enzymatic reactions, including those involved in its own synthesis (Bloch, 1977; Wititsuwannakul & Kim, 1977), and the polymethyl polysaccharides are effective carriers and sequestrators of this lipid. A broader function for the polymethyl polysaccharides is suggested, however, by the fact that two types are found in the same cell and that one of them is subject to variable degrees of acylation. The effect of acylation appears to be to stabilize the methylglucose polysaccharide in a helical conformation, whereas the methylmannose polysaccharide is more flexible and assumes a completely helical conformation only when in a complex with lipid (Yabusaki et al., 1979; Maggio, 1980).

With regard to the role of acylation in MGLP, the short-chain neutral acyl groups are expected, like methylation, to enhance the nonpolar interaction of the polysaccharide with its host fatty acid. The octanoyl group, in contrast, may serve to raise the interreaction energy required for complex formation between MGLP and an extraneous lipid. Free octanoic acid and octanoyl-CoA give poor complexes with MMP or MGP because the alkyl chain is too short (Yabusaki & Ballou, 1978), but an octanoyl group esterified to MGP might by its proximity to the polysaccharide chain become a very effective complexing agent. According to this idea, only a long alkyl chain with good complexing ability would be able to displace the octanoyl group, which thus would confer on the MGLP molecule a level of discrimination not present in MMP. Finally, it is possible that the acidic succinyl groups serve to increase the solubility of MGLP–lipid complexes or that they are involved in the interaction of such complexes with cations, a protein, or the membrane.

Acknowledgments

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