

SPECIFIC INTERACTION OF MYCOBACTERIAL POLYMETHYLPOLYSACCHARIDES
WITH LONG-CHAIN FATTY ACIDS AND ACOYL-COENZYME A DERIVATIVES

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Abstract - Mycobacteria synthesize two predominantly α 1 \rightarrow 4-linked glycans, one composed of 3-O-methyl-D-mannose and the other of 6-O-methyl-D-glucose. These substances form complexes with long-chain fatty acids in which the lipid is included in the interior of the helically coiled polysaccharide. The dissociation constant obtained by fluorimetric titration with parinaric acid is 0.4 μ M, and parinaric acid is displaced from the complex by palmitoyl-coenzyme A, which indicates that the latter binds even more tightly. Proton NMR studies confirm that the polysaccharide in the complex has a helical conformation and that the fatty acid is included in a specific orientation. It is postulated that these polymethylpolysaccharides serve as lipid carriers in the cell and facilitate the synthesis of the unusually large fatty acids that are characteristic of mycobacteria.

INTRODUCTION

As the variety of living organisms studied in detail increases, there becomes apparent an ever increasing number of specialized biochemical roles for unusual carbohydrate derivatives. Perhaps the most dramatic confirmation of this statement is the variety of oligosaccharide structures found on glycoconjugates that seem to be involved in cell-cell recognition phenomena (Ref. 1). These oligosaccharides, assemblies of 5-20 monosaccharides, are often rich in mannose and N-acetylglucosamine, and, depending on the organism, also may contain galactose, sialic acid, fucose, glucuronic acid or other sugars.

Microorganisms are probably the richest source of diversity for molecules of this type because each organism is highly specialized to fit one of the many different environmental niches available. In this regard, mycobacteria, the causative agents of diseases such as tuberculosis and leprosy, have attracted an inordinate share of the attention of chemists and biologists. From the time of R. J. Anderson who studied their complex lipids (Ref. 2), to E. Lederer who analyzed the mycolic acids and the adjuvant properties of the cell walls (Ref. 3), to A. F. Brodie who has used this bacterium as a model for studies on oxidative phosphorylation (Ref. 4), to K. Bloch who has characterized the fatty acid synthetases (Ref. 5), mycobacteria have had a wide appeal to scientists interested in natural products.

One obvious reason why this should have been so is the importance mycobacteria have as human pathogens and, even though the scourge of TB no longer inflicts its pain on Western societies as it once did, the disease is still endemic to many cultures. A thesis one can argue is that the success of *Mycobacterium tuberculosis* as a pathogen is closely related to its development of a specialized class of polysaccharide cofactors that serve as lipid carriers and allow the bacterium to synthesize a cell wall of unusually high lipid content (Ref. 6). Such a cell wall could be a major deterrent to the action of macrophages and their lysosomal enzymes, so that low-level infections by mycobacteria could survive the onslaught of the mammalian immune system. Furthermore, the correlation that is observed between the size of the mycolic acids found in the cell wall and pathogenicity supports the idea that the more nonpolar and impervious the wall the better adapted is the organism to survive in the human body.

POLYMETHYLPOLYSACCHARIDE STRUCTURES

What is the special class of polysaccharide regulators of lipid metabolism? We call them polymethylpolysaccharides (abbreviated PMPS) because they are composed largely of mono-O-methylhexoses, 6-O-methyl-D-glucose in one instance (Ref. 7) and 3-O-methyl-D-mannose in the other (Ref. 8). Both of these unusual polymers were discovered when coworkers in my laboratory observed these sugars in acid hydrolysates of extracts of *Mycobacterium smegmatis*.

Interestingly, to this time this is the only report of the natural occurrence of 6-O-methylglucose. The structures of the PMPS are shown in Fig. 1 and 2.

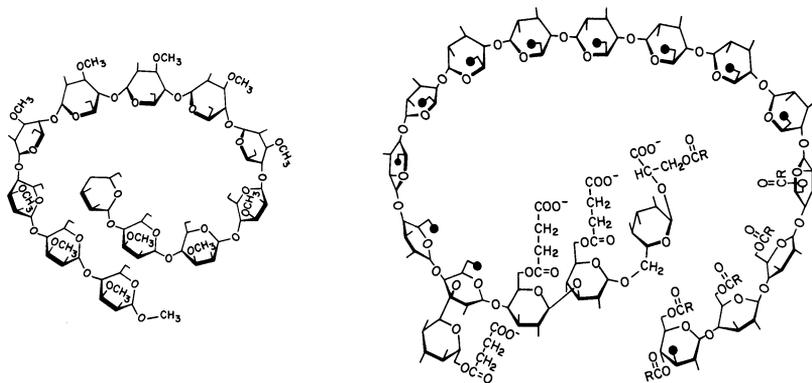


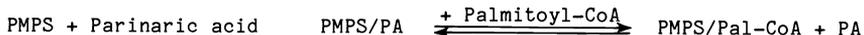
Fig. 1 (left). Structure of the 3-O-methylmannose polysaccharide (MMP) homolog with twelve sugar units. All linkages are α 1-4.

Fig. 2 (right). Structure of the 6-O-methylglucose lipopolysaccharide (MGLP). The closed circles represent methyl ether groups. Deacylation yields the 6-O-methylglucose polysaccharide (MGP).

Shortly after our report on the structure of MGLP, Konrad Bloch observed that the PMPS were able to substitute for a heat-stable factor that stimulated fatty acid synthesis by the isolated fatty acid synthetase complex from *M. smegmatis* (for a review see Ref. 5). Palmitoyl-coenzyme A is a potent inhibitor of the enzyme complex and the PMPS relieved this inhibition by sequestering the lipid much as amylose is able to complex fatty acids (Ref. 9). The substance of my following report deals with studies we have carried out to determine the dissociation constant for this interaction and with evidence we have obtained that the PMPS undergo a well-defined conformational change when they form a complex with lipids.

POLYMETHYLPOLYSACCHARIDE-LIPID DISSOCIATION CONSTANTS

The system we have employed to evaluate dissociation constants is represented in the following expression. β -Parinaric acid (PA), all trans 9,11,13,15-octadecatetraenoic acid, is a



good analog of stearic acid that, because of the 4 conjugated double bonds, shows a strong enhancement of fluorescence in apolar solvents (Ref. 10). Assuming that the complex between PMPS and PA would result from inclusion of the lipid in the nonpolar interior of a coiled polysaccharide chain, by analogy with amylose or the cyclodextrins (Ref. 11), we expected to obtain a typical fluorimetric titration curve from which a dissociation constant could be evaluated. As illustrated in Fig. 3, such a result was obtained from which we determined that a 1:1 complex was formed with a K_d of 0.4 μ M (Ref. 12). The findings illustrated in Fig. 3 are a bit surprising in that one might not expect such tight binding of a lipid by an oligosaccharide, and, in fact, the dissociation constants for cyclodextrin complexes with aromatic compounds such as *p*-nitrophenol are about 1000-fold larger (Ref. 13). It is also striking that palmitoyl-CoA displaces the parinaric acid probe from the complex so effectively, which shows that the CoA moiety must enhance the binding, probably by a hydrophilic interaction with the hydroxyl groups on the surface of the coiled polysaccharide. Coenzyme A derivatives have been shown to have a hair-pin conformation in aqueous solution (Ref. 14), and this conformation, if retained in the complex, would facilitate complexation.

The determination of precise physical constants on very low concentrations of lipids such as palmitoyl-CoA are open to error owing to the tendency of the lipids to form micelles, to adsorb to surfaces and to decompose, so we have sought confirmation of the determined dissociation constant by an alternative procedure. This involved a study of the effect of PMPS on the activity of a thioesterase we isolated from *M. smegmatis* (Ref. 15). The enzyme is specific for long-chain acyl-CoAs and it hydrolyzes them to CoA and free fatty acid. With palmitoyl-CoA, the V_{max} is 230 μ moles of substrate hydrolyzed/min/mg of enzyme protein and the K_m is 5 μ M for micellar substrate and 9 μ M for monomeric substrate. If the K_d we determined for the PMPS/PA complex is correct, then the PMPS should be good inhibitors of the thioesterase reaction. As documented in Table I, the reaction is strongly inhibited by a

PMPS that is capable of complexing the substrate. Note that hydrolysis of lauroyl-CoA, a substrate that forms a weak complex with MGP, was not affected. This, and the observation that a noncomplexing polysaccharide analog (AG-MGP) was not inhibitory, demonstrates that the inhibition is due to a complexing of the substrate by the PMPS and not to an interaction of the PMPS with the enzyme.

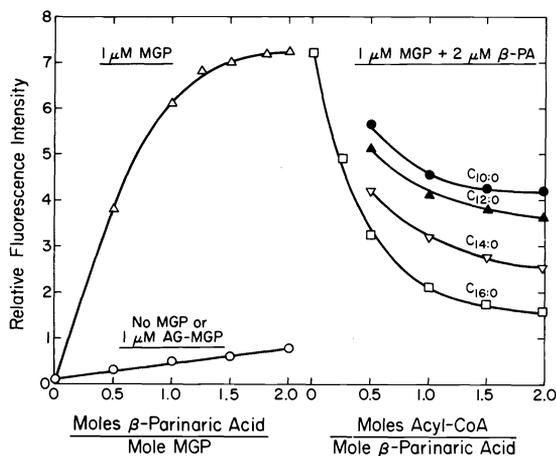


Fig. 3. Titration of methylglucose polysaccharide (MGP) with β -parinaric acid (PA), and displacement of PA from the complex by acyl-CoA derivatives of different chain lengths. On the left, $1 \mu\text{M}$ MGP was titrated to $2 \mu\text{M}$ PA. On the right, this solution was back-titrated to $2 \mu\text{M}$ concentrations of various acyl-CoAs. AG-MGP is amylase/glucoamylase-digested MGP.

TABLE 1. Effect of MGP on the hydrolysis of acyl-CoA derivatives

Palmitoyl-CoA concentration (μM)	Lauroyl-CoA concentration (μM)	MGP concentration (μM)	Initial rate $\mu\text{mol}/\text{min}/\text{mg}$ thioesterase
2			13.9
4			29.8
10			42.5
10		2	39.7
10		4	30.6
10		10	12.0
10		20	3.6
	10	0	7.5
	10	8	7.4

STRUCTURAL REQUIREMENTS FOR COMPLEXATION

The structural requirements for good complex formation between PMPS and acyl-CoAs are consistent with the kind of complex that is postulated. There is a specific dependence of the interaction both on the length of the fatty acid and the polysaccharide (Ref. 12). The PMPS must have an $\alpha 1 \rightarrow 4$ -linked sequence of at least 12 sugars and the optimum fatty acid chain length is about 16 carbon atoms. It may be significant that 12 sugars are just sufficient for two complete turns of the helical conformation, as in α -cyclodextrin, and that palmitic acid would be just long enough to reach from one end to the other of the tightly coiled dodecamer homolog of MMP.

The polysaccharide chain length requirement for complexation has been demonstrated for both MMP and MGP. From *M. smegmatis* extracts, one can isolate MMP homologs with 5 to 15 sugar units (Ref. 16). The shorter oligosaccharides appear to be biosynthetic precursors and the homologs of 12 to 15 sugars are thought to accumulate in the cell because they develop the ability to complex with lipids, which prevents further elongation. Comparison of these homologs demonstrated that the MMP with 11 sugars complexed parinaric acid poorly whereas those with 12-15 sugars had good complexing ability (Ref. 12).

With MGP, biosynthesis occurs by a different pathway, and smaller precursors do not occur in the cell (Ref. 17). The presence of 3 unmethylated α 1 \rightarrow 4-linked glucose units at the non-reducing end of MGP provides a point of attack for amylases, however, and through the combined action of α -amylase and glucoamylase 4 hexose units can be removed. This reduces the α 1 \rightarrow 4-linked section of MGP from 15 to 11 sugars and the modification simultaneously eliminates its lipid-binding ability (Fig. 3).

POLYMETHYLPOLYSACCHARIDE CONFORMATIONS

MGP, by virtue of its carboxyl group on the glyceric acid unit at the reducing end of the chain, can be coupled to tryptophan in amide linkage, thus incorporating a useful probe for studying conformation. We were surprised to observe that the circular dichroism spectrum of MGP-Trp showed 3 bands of negative ellipticity indicative of a structured or restricted tryptophan residue (Ref. 12). A second surprise was that this complex spectrum was absent in the parallel derivative made with AG-MGP. These results suggest that there is some ordered structure in MGP-Trp, even in the absence of fatty acid, and that this structure is required for complexation of the lipid since AG-MGP lacks both the ability to bind fatty acids and to affect the CD spectrum of tryptophan.

Although the enhancement of fluorescence of parinaric acid on titration of PMPS is a strong indication that the fatty acid is included in the interior of the coiled polysaccharide, we have sought more direct evidence for this model. Such evidence comes from proton NMR studies on both components of the system (Ref. 18). First, I will discuss the effects of complexation of MMP and, second, the changes that occur in palmitic acid. The anomeric protons of the 3-O-methylmannose units in MMP are intrinsic probes of chain conformation because the chemical shift of H-1 is sensitive to the bonding angles between two such pyranose sugars in α 1 \rightarrow 4 linkage (Ref. 19). As reference chemical shifts we have used those of the compounds in Table 2. The nonaglucoamylose is considered to exist as a random coil whereas α -cyclodex-

TABLE 2. Proton chemical shifts of reference oligosaccharides

Compound	H-1 chemical shift (δ)
α -Cyclodextrin	5.039
β -Cyclodextrin	5.051
γ -Cyclodextrin	5.103
Nonaglucoamylose	5.380

trin should have a fixed conformation with H-1 and H-4' nearly eclipsed. As the cyclodextrin ring is enlarged, the greater flexibility is associated with a shift of the H-1 resonance back toward that of the acyclic nonaglucoamylose.

The NMR spectra, taken at 180 MHz, for MMP and its complex with palmitic acid are shown in Fig. 4. The H-1 protons for the 10 methylmannose units in the middle of the MMP chain appear at about 5.22 ppm, and 8 of these shift upfield to about 5.0 ppm in the complex. We interpret this to mean that the polysaccharide has changed conformation dramatically and is tightly coiled around the included fatty acid.

If the palmitic acid is included in the apolar interior of the coiled MMP, the majority of the methylene protons should be deshielded by van der Waals effects (Ref. 20), and this is observed (Fig. 5). Both the terminal methyl and the majority of the methylenes resonate at lower field in the complex. Interestingly, the α -methylene protons are affected differently, and one explanation for their upfield shift is that the pKa of the carboxyl group has been changed so that the acid is more highly dissociated in the complex (Ref. 21). What is the disposition of the mannose methyl groups in the complex? If they are closely associated with the fatty acid one would expect them to experience a similar deshielding, and this is observed (inset D in Fig. 4). Although all of the methyl signals have not been resolved, the majority do show a downfield shift consistent with their environment becoming less polar in the complex. One anomaly is apparent, however, in that the methyl aglycon resonance is shifted upfield about 0.07 ppm. One explanation for this effect is that the palmitic acid is oriented in the coiled MMP with its carboxyl group juxtaposed to the methyl aglycon such that the latter is in the shielding cone of the carbonyl group (Ref. 22). This arrangement is reasonable because, in a left-handed helix, the leading edge of the coil from the reducing end of the chain is coated with methyl groups that could provide a favorable apolar region of entry for the methyl end of the fatty acid.

All of our studies are consistent with the model shown in Fig. 6 for the complex between MMP and palmitic acid. Because distinct signals are not seen for the complexed and uncomplexed polysaccharide, the fatty acid must be exchanging rapidly. From the difference in H-1

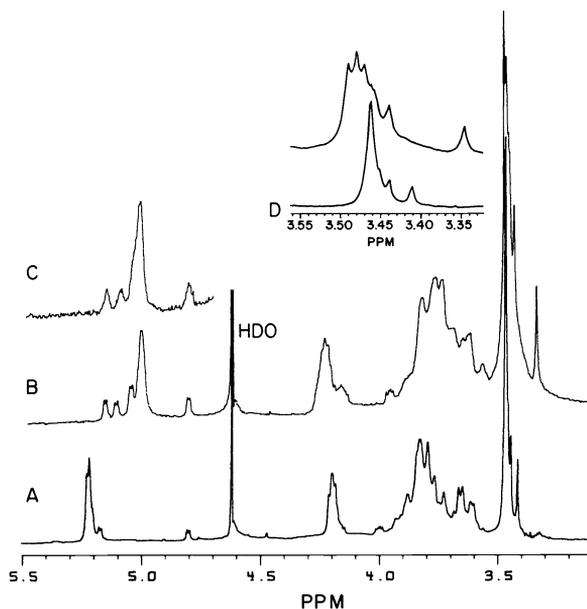


Fig. 4. Proton NMR spectra of MMP and its complex with palmitic acid. A is free MMP, B is the complex of MMP with palmitic acid, C is the complex of MMP with stearic acid, and D is an expanded spectrum for the methyl protons of MMP (bottom) and its complex with palmitic acid (top) in which the methyl aglycon has shifted from 3.41 to 3.34 ppm.

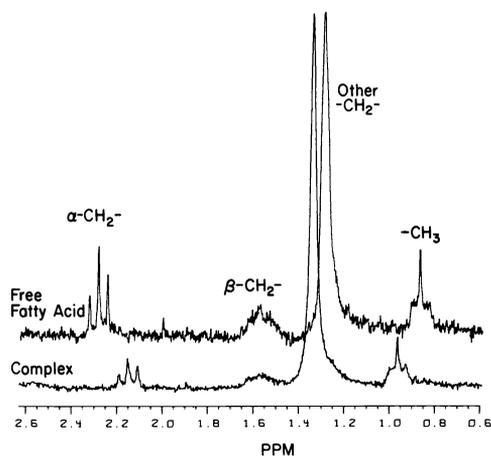


Fig. 5. Proton NMR spectra of free decanoic acid and of the MMP/palmitic acid complex. Decanoic acid was used as a reference because free palmitic acid is too insoluble for the NMR measurement.

chemical shifts between free and complexed MMP, we calculate (Ref. 23) that the slow rate of dissociation of the complex must be about 200 sec^{-1} . If this rate and the K_d of $0.4 \mu\text{M}$ are used to calculate a rate of formation, the value of $5 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ is obtained. This value is near that of diffusion controlled processes and is similar to the forward rate constant determined by temperature jump experiments for the association equilibrium of α -cyclodextrin and *p*-nitrophenylate (Ref. 13).

Conclusions: It is clear that the amylose-like structure of the PMPS, in conjunction with the methylation, confers on these molecules the unique property of being able to form very stable complexes with long-chain fatty acids. That these molecules play an important role in lipid metabolism in mycobacteria seems certain. The high acyl-CoA content of mycobacterial cells is in sharp contrast to the virtual absence of such compounds in *Escherichia coli* (Ref. 24, p. 181). Moreover, the latter contain a very active acyl-CoA thioesterase that is postulated to have as one function the maintenance of a low cytoplasmic level of palmitoyl-CoA,

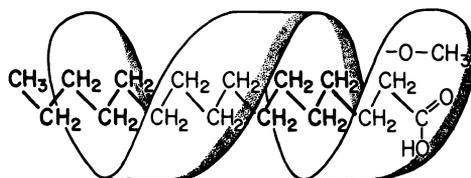


Fig. 6. Schematic representation of MMP/palmitic acid complex. The fatty acid is oriented with its carboxyl group near the methyl aglycon of the MMP. The shaded area along one edge of the ribbon represents the 3-O-methyl groups.

which is a potent inhibitor of many metabolic reactions. The evolution of a bacterium with the ability to make PMPS and use them as lipid carriers allows the cell to function with a much higher apparent cytoplasmic concentration of palmitoyl-CoA and, in turn, to make the very large and insoluble lipids that are found in the cell wall. In fact, this chance development may define the genus *Mycobacterium* because we have found these compounds in all but one bacterial strain classified in this genus, and MMP in a modified form has been found in only one bacterium outside this genus (Ref. 25).

If the α 1 \rightarrow 4-glucan structure can be used so effectively by mycobacteria, is it reasonable to expect that higher organisms would have ignored this structural feature as a mechanism by which to regulate glycogen metabolism? Recent experiments on glycogen synthetase regulation by palmitoyl-CoA show that this lipid inhibits glycogen synthesis by complexing both to the enzyme and the glycogen (Ref. 26). Even though the α 1 \rightarrow 4-linked sections of glycogen are considered too short to form very tight complexes with fatty acids, the interaction may be sufficiently stabilized by some unusual feature of the large glycogen molecule such that this interaction could become physiologically important.

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