Proc. Natl. Acad. Sci. USA
Vol. 77, No. 5, pp. 2582–2586, May 1980
Biochemistry

Structure of a mycobacterial polysaccharide–fatty acyl-CoA complex: Nuclear magnetic resonance studies

(^{13}C relaxation times/ligand-induced conformational change)

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Communicated by Konrad Bloch, February 19, 1980

ABSTRACT **MMP, a linear α1→4 linked polymer of 3-*O*-methylmannose, regulates the fatty acid synthetase from *Mycobacterium smegmatis* by forming stoichiometric complexes with the long-chain acyl-CoA synthetase products. In agreement with previous proposals [Bloch, K. (1977) in *Advances in Enzymology and Related Areas of Molecular Biology*, ed. Meister, A. (Wiley, New York), Vol. 45, pp. 1–84], nuclear magnetic resonance studies show that the polysaccharide, a random coil in its free form, undergoes a major conformational transition upon enclosing long-chain acyl-CoA. The polysaccharide, probably in helical conformation in the complexed form, interacts with both the paraffinic chain and the CoA moieties of the included fatty acyl thioester.**

Mycobacterium smegmatis produces two types of unusual polysaccharides containing repeating segments (10–12 residues) of 6-*O*-methylglucose or 3-*O*-methylmannose (1–4). These intracellular polysaccharides profoundly affect the fatty acid synthetase from the same organism (4, 5). They increase the overall rate of *de novo* synthesis markedly (4), shift the chain length of products (6), and prevent inhibition of the enzyme by endproducts (7). These effects can be mimicked qualitatively by α- and β-cyclodextrins, cyclic glucans of six and seven residues, respectively, which form inclusion complexes with fatty acids and acyl-CoA derivatives (8–10). Certain methylated derivatives (notably 2,6-heptakis-di-*O*-methyl-β-cyclodextrin) mimic the effects of the mycobacterial polysaccharides even more effectively than the parent compounds (8). It was proposed that similar inclusion complexes result from the interaction of long-chain acyl-CoA and the mycobacterial polysaccharides (11). Because complexation is enhanced when cyclodextrins are methylated, it was further suggested that the lipophilic regions of both the methylmannose-containing (MMP) and methylglucose-containing polysaccharides are likely sites for the binding of fatty acyl chains (7, 8). Subsequently, evidence for the formation of stoichiometric complexes between MMP and acyl-CoA derivatives was obtained by column chromatography (12). Moreover, a fatty acyl-CoA-induced conformational change in the polysaccharide was indicated by the drastic change in the optical rotation of MMP upon addition of palmitoyl-CoA (11).

To account for the much tighter binding of acyl-CoA derivatives than of free fatty acids, it was further proposed that the CoA moiety contributed to complexation by interacting with hydrophilic regions of the polysaccharide (11). It was pointed out that appropriate rotation of the glycosidic bonds of MMP yields a helical conformer displaying an interior channel of appropriate size and hydrophobicity (inward-facing methyl groups) for interaction with paraffinic chains. Model building added final refinements and led to the following conclusion. On contact with acyl-CoA, MMP undergoes a conformational change from random coil to helix; the 3-*O*-methyl groups provide a hydrophobic channel that accommodates the paraffinic chain of acyl-CoA. The CoA moiety folds back in hairpin fashion and interacts with the hydrophilic exterior of the polysaccharide helix (5, 11).

I now present nuclear magnetic resonance studies in support of the postulated conformational changes in MMP that result from the interaction with acyl-CoA. These data also support the involvement of the CoA moiety in the complexation process.

EXPERIMENTAL

MMP was purified from *M. smegmatis* as described (4), except that the final product was extracted with chloroform to remove residual lypophilic impurities. Molar ratios of 3-*O*-methylmannose:mannose in the purified polysaccharide were determined by gas chromatography of trimethylsilyl ether derivatives (13). CoASH and some of the acyl-CoA derivatives were products of P-L Biochemicals. Other acyl-CoA derivatives were synthesized from CoASH by using the *N*-hydroxysuccinimide ester as acylating agent (14).

NMR studies were performed at 37°C in 0.1 M phosphate buffer, pH 6.6/1 mM EDTA in 99% ^2H_2O, duplicating conditions for mycobacterial fatty acid synthetase assays, except when noted. All experiments were done in the Fourier mode on solutions of 1–10 mM concentration; chemical shifts and relaxation times are concentration independent over this range. Assignments of NMR resonance lines were made by analogy to model compounds and confirmed by various double resonance experiments. Chemical shifts are reported relative to the following: ^{13}C-external $(CH_3)_4Si$ coaxial capillary, ^{31}P-external H_3PO_4 (85%) coaxial capillary, and ^1H-internal $(CH_3)_3Si-CH_2CH_2SO_3Na$. Actual referencing was sometimes indirect via lock resonance or an appropriate internal standard. With the exceptions of OCH_3 groups, whose relaxation times were determined by the progressive saturation technique (15), ^{13}C spin-lattice relaxation times (T_1) were measured by inversion-recovery (16), observing the usual caveats (17). At least seven τ values were used for each T_1 determination and intensities were computer-fitted by least squares to an exponential curve by using a two- or three-parameter fit. ^{13}C-{^1H} nuclear Overhauser effects (NOEs) were measured by gated noise decoupling (18). (NOE is defined as the ratio of intensities with and without proton decoupling.) Recycle delays were at least 5 T_1 for T_1 measurements and 8 T_1 for NOE measurements. All T_1 and NOE measurements were done in duplicate; estimated errors are ±10% and ± 20%, respectively. Buffers were degassed by freeze-pump-thaw before solutions were prepared.

Hydrolysis of acyl-CoA was assayed by 5,5′-dithiobis(2-ni-

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Abbreviations: MMP, the 3-*O*-methylmannose polysaccharide from *M. smegmatis*; NOE, nuclear Overhauser effect; T_1, spin-lattice relaxation time.

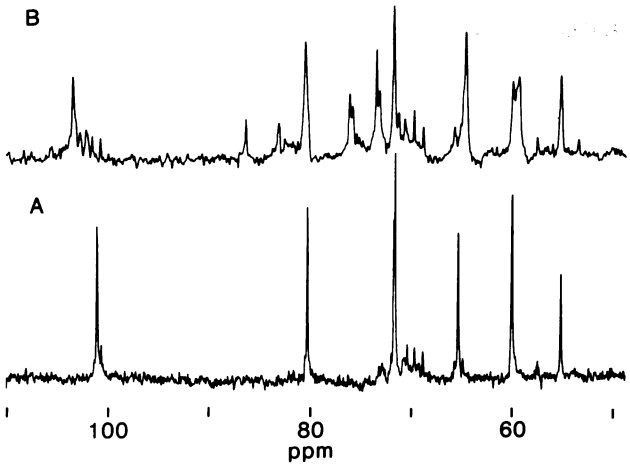

FIG. 1. Sixty-eight megahertz ^{13}C NMR spectrum of MMP: (*A*) free; (*B*) in the presence of excess palmitoyl-CoA. Plots are from 110 to 50 ppm. Chemical shifts are reported in Table 1.

trobenzoic acid) (19). The pH meter readings are uncorrected. Methyl-α-D-3-*O*-methylmannose was synthesized by minor modification of a published procedure (20).

RESULTS

The ^{13}C NMR spectrum of MMP (Fig. 1*A*) is consistent with the chemically (3, 21) established structure—a linear α1→4-linked polymer of 3-*O*-methylmannose.* A single sharp resonance can be assigned to each carbon of the monomer unit (C-4 and C-5 are barely resolved at 68 MHz) of the central chemically equivalent methylhexose residues of the polymer. Some resonances from the terminal residues of the molecule are also resolved. The one-bond coupling of 172 Hz between anomeric carbon and proton confirms that the linkage is α (22). The ^{13}C chemical shifts of MMP (Table 1) are similar to those of a model monomer (methyl-α-D-3-*O*-methylmannopyranoside) and are not significantly perturbed by conditions (23) disruptive of polysaccharide conformation (temperature, pH, or organic solvents).

In the presence of excess palmitoyl-CoA, the ^{13}C resonances of MMP are all shifted, but to varying degrees (Figs. 1 and 2; Table 1). The change in chemical shift is most pronounced for the anomeric carbon C-1, whose major peak, contributed by about seven of the sugar residues, is shifted downfield by more than 2 ppm. For the other anomeric carbons the increments are smaller; clearly, complexation with $C_{16:0}$-SCoA does not affect all regions of the polysaccharide chain alike. Spin-lattice relaxation times (T_1), linewidths ($W_{1/2}$), and ^{13}C-{^1H} NOEs for MMP, both free and complexed with acyl-CoA, are summarized in Table 2.

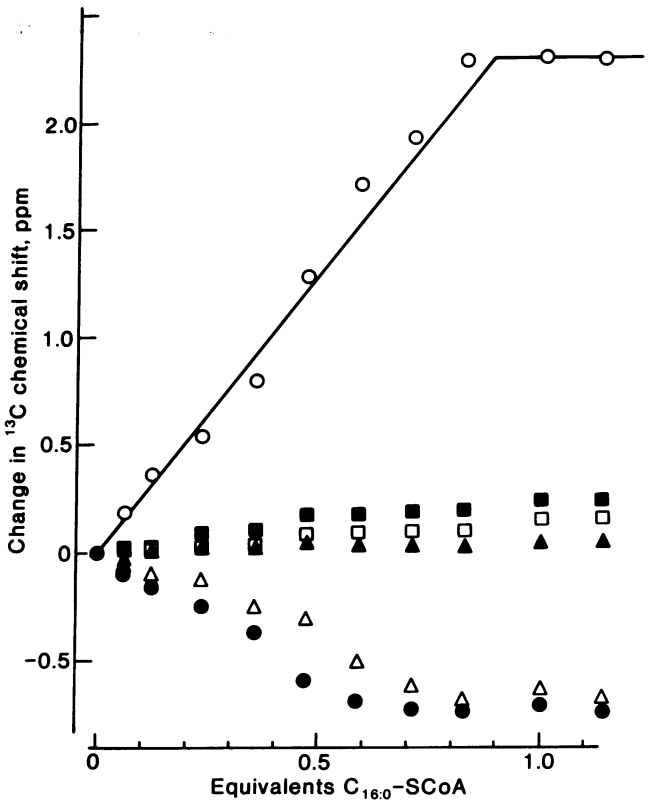

FIG. 2. Changes in the chemical shifts of MMP carbons induced by binding palmitoyl-CoA. O—O, C-1 (major peak); ●, C-2; ■, C-3; □, C-4 and C-5; △, C-6; ▲, OCH_3.

For purposes of comparison, α-cyclodextrin has been similarly studied by ^{13}C NMR, both as such and in its complex with palmitoyl-CoA. Relaxation times, linewidths, and NOEs are presented in Table 3. From the data in Tables 2 and 3, rotational correlation times of the polysaccharides in both free and complexed form can be calculated (Table 4).

Addition of palmitoyl-CoA also changes the ^1H NMR spectrum of MMP, although the effects are considerably smaller

* Actually, MMP as isolated consists of a small family of similar polysaccharides whose major structural feature is a sequence of 10–12 α1→4-linked residues of 3-*O*-methylmannose. Some unmethylated mannose residues are also present; for a complete discussion, see ref. 3. MMP used in this work varied in 3-*O*-methylmannose:mannose ratios between 10:1 and 14:1, with no detectable difference in biological activity (i.e., effects on fatty acid synthesis).

Table 1. ^{13}C chemical shifts of MMP (ppm)

Carbon	Free MMP	MMP–palmitoyl-CoA complex	MMP–palmitic acid complex*
C-1	101.24	103.54	103.01
C-2	66.36	65.65	66.03
C-3	80.83	81.08	81.02
C-4	72.47†	72.49‡	72.49‡
C-5	72.34†	72.49‡	72.49‡
C-6	61.07	60.38	60.92
OCH_3	56.31	56.36	56.41

* In ^2H_2O.
† Assignments may be reversed.
‡ Unresolved at 68 MHz.

Table 2. ^{13}C relaxation times, NOEs, and linewidths for free and complexed* MMP

MMP	T_1, msec 25 MHz	T_1, msec 68 MHz	NOE 68 MHz	$W_{1/2}$, Hz 68 MHz
Free ⟨C-1–C-5⟩† sugar ring	131	183	2.1	10
C-6 primary hydroxyl	93	129	2.6	15
OCH_3	607	663	1.6	10
Complexed ⟨C-1–C-5⟩ sugar ring	61	113	1.3	16
C-6 primary hydroxyl	52	64	1.7	26
OCH_3	434	464	1.4	14

* In the presence of 1.3 equivalents of palmitoyl-CoA.
† Within experimental error, values for the sugar-ring carbons are equal. Their average is reported and used for calculations.

Table 3. ^{13}C relaxation times, NOEs, and linewidths for free and complexed* α-cyclodextrin

α-Cyclodextrin	T_1, msec 25 MHz	T_1, msec 68 MHz	NOE 68 MHz	$W_{1/2}$, Hz 68 MHz
Free ⟨C-1–C-5⟩†				
sugar ring	155	194	2.3	3.1
C-6 primary hydroxyl	109	113	2.4	4.7
Complexed ⟨C-1–C-5⟩				
sugar ring	88	119	1.7	5.0
C-6 primary hydroxyl	51	70	1.8	6.7

* In the presence of 0.5 equivalent of palmitoyl-CoA.
† See footnote to Table 2.

than those observed for ^{13}C. Although the ^1H spectrum of the MMP–palmitoyl-CoA complex is substantially the same as that reported recently for the MMP–palmitic acid complex (25), the ^{13}C spectra reveal significant differences between the two (Table 1).

Complexation with MMP also induces changes in the chemical shifts of ^1H, ^{13}C, and ^{31}P nuclei of the guest palmitoyl-CoA (Table 5 and Fig. 3). The induced shifts are a linear function of MMP concentration; kinetics of complexation are fast on the NMR time scale. Interpretation of these data is complicated by the fact that the induced shifts are the result of two processes: disruption of the palmitoyl-CoA micelles, followed by complexation of the monomeric acyl-CoA by MMP. The former process cannot be directly studied by NMR because the critical micelle concentration (about 4 μM) lies far below the limits of detection of the technique (10). [The dissociation constant of the complex is about 0.1 μM (27).] Studies of MMP–palmitoyl-CoA interaction below the critical micelle concentration require other methods (27). For purposes of comparison, data for CoASH itself have been included; the coenzyme neither forms micelles† nor interacts with MMP (11).

Table 4. Rotational correlation times (τ_c) of MMP and α-cyclodextrin in the free and complexed form (psec)

	Free	Complexed
MMP ⟨C-1–C-5⟩		
sugar ring	360	1170
C-6 primary hydroxyl	220	520
OCH_3	25	38
α-Cyclodextrin ⟨C-1–C-5⟩		
sugar ring	330	640
C-6 primary hydroxyl	260	540

The τ_c values were calculated from the data in Tables 2 and 3 according to ref. 24. The extreme narrowing condition does not apply to all of these data. We have assumed completely dipolar relaxation, isotropic motion of a rigid C-H vector, and a C-H bond distance of 1.10 Å. The particular τ_c values above have been calculated from 68 MHz T_1 data for the free and 25 MHz T_1 data for the complexed polysaccharide in each case. Within experimental error, all three parameters (25 MHz T_1, 68 MHz T_1, and 68 MHz NOE) give identical values for τ_c. Although each of these measurements is independent, at least two are required to uniquely define τ_c because T_1 is not a monotonic function of τ_c. Linewidths are not a reliable source of information about τ_c because slight chemical shift inequivalence (undoubtedly the case for MMP) and field inhomogeneity contribute more to $W_{1/2}$ than does T_2. The assumptions of completely dipolar relaxation and isotropic motion probably do not apply to the *O*-methyl groups.

Table 5. Chemical shifts (ppm) of palmitoyl-CoA and CoASH* in the presence and absence of MMP

Resonance	Micellar CoASR, $^{13}C/^1H$	MMP–CoASR complex, $^{13}C/^1H$	CoASH with or without MMP, $^{13}C/^1H$
Fatty acid			
O ‖ C—S	201.09	199.91	
$(CH_2)_n$ major	29.88/1.27	30.46/1.36	
Adenine			
A2	151.51/8.15	152.79/8.29	153.05/8.28
A8	140.29/8.50	140.02/8.56	140.01/8.54
Ribose			
R1	87.26/6.14	86.82/6.19	86.58/6.17
R4	83.27	83.59	83.89
R2	74.12†	75.79†	74.15
R3	74.05†	75.51†	73.89
Pantetheine			
P1	72.21	72.04	71.90
P3	74.25	74.20	74.28
P2Me′	21.24/0.93	21.08/0.90	20.85/0.86
P2Me″	18.31/0.75	18.20/0.75	18.22/0.74
Differences			
|P2Me′ − P2Me″|	2.93/0.18	2.88/0.15	2.63/0.12
|A2 − A8|	11.22/0.35	12.77/0.27	13.04/0.26
|R1 − R4|	2.99	2.23	2.69

* Nomenclature of CoA is from ref. 26.
† Assignments may be reversed.

The large change in the ^{31}P chemical shift of the 3′-phosphate of palmitoyl-CoA upon complexation reflects a difference in the pK_a of this group in the micelle and in the MMP complex (Fig. 3*B*).

DISCUSSION

The sensitivity of ^{13}C (and, to a lesser extent, ^1H) chemical shifts to conformation (distribution of rotamers) is well established for polypeptides (29–32) and polysaccharides (33–37). However, no adequate theoretical treatment exists for the general case correlating changes in chemical shifts with specific conformational transitions. Empirical generalizations are justified, therefore, only if observed chemical shifts can be independently assigned to known configurations. This has been done in certain cases for both polypeptides and polysaccharides; downfield shift of C-α in the former case and C-1 in the latter appears to be associated with the coil→helix transition in the examples studied (29–32, 34–36).

Motion-dependent relaxation parameters are a much more reliable barometer of conformational change than chemical shifts because no empirical calibration is required. For ^{13}C nuclei with directly bonded protons, the overwhelmingly dominant relaxation mechanism is dipolar and, from relaxation times (T_1, T_2) under decoupling conditions and ^{13}C-{^1H} NOE, rotational correlation times (τ_c) of the C-H vectors can be calculated (24, 38). For details of the calculations, see Table 4.

Theoretical calculations (39) have pointed to a random coil→helix transition when the polysaccharide MMP complexes long-chain acyl-CoA. Similarly, the marked decrease in the optical rotation of MMP (11, 25) provides clear evidence for a conformational change from a less to more ordered structure as the complex is formed. Both the sign and magnitude of this

† Although CoASH can form aggregates in solution through base stacking, their contribution is negligible under the experimental conditions (28).

shift in optical rotation are suggestive of a binding-induced conformation transition from random coil to V-amylose-like helix (39). Our NMR studies are entirely consistent with these interpretations.

The insensitivity of the ^{13}C chemical shifts of free MMP to denaturing conditions implies that the polysaccharide as such assumes a random coil conformation. Comparison of the ^{13}C chemical shifts of free MMP under a variety of denaturing conditions with those of MMP complexed with long-chain acyl-CoA suggests that no more than a few percent of the restricted conformation found in the complex is present in free MMP at 37°C in aqueous solution. Consistent with this conclusion, the carbon T_1 values approximately equal those of a random coil glucan of about the same molecular weight (34). Comparison of the T_1 values of α-cyclodextrin and MMP (Tables 2 and 3) shows that although the molecules differ by 2.5-fold in molecular weight, their respective sugar residues have about the same mobility; clearly MMP has a much less restricted conformation.

The changes in chemical shifts of MMP carbons seen when the polysaccharide complexes acyl-CoA are largest (about 2.3 ppm downfield) at the conformationally sensitive anomeric carbons C-1 (Fig. 2), much larger than the change induced at C-1 (or any other carbon) of α-cyclodextrin upon binding the same guest. It is also larger than the difference in C-1 chemical shifts of amylose and α-cyclodextrin and larger than the changes in chemical shift of C-1 of amylose induced by denaturants (NaOH or dimethyl sulfoxide) (33, 37). A downfield shift of similar magnitude at C-1 (34) is associated with a coil →helix transition in a β1→3 glucan. The methylglucose-containing lipopolysaccharide MGLP-III (1, 2), another active stimulant of the fatty acid synthetase (11), also displays a downfield shift at C-1 as it complexes CoASR. Other relatively large complexation-induced shifts in MMP carbon resonances are at C-2 and C-6.‡

Upon complexation of palmitoyl-CoA, the T_1 values of the MMP carbons decrease markedly (Table 2), indicative of a substantial decrease in mobility. The consequent increase in τ_c for MMP is much larger than the corresponding increase in τ_c for complexation of CoASR by α-cyclodextrin, a cyclic molecule that can change conformation only slightly (Table 4). The decrease in mobility of α-cyclodextrin on complexing $C_{16:0}$-SCoA is of about the same magnitude as that observed for other guest molecules and indicates residual motion in the complex (40). Because the α-cyclodextrin–CoASR complex chosen for comparison has a stoichiometry of 2 α-cyclodextrin to 1 acyl-CoA, the molecular weight of the cyclodextrin and MMP complexes is nearly the same (about 3100 and 3400, respectively). With 1:1 polysaccharide–acyl-CoA complexes, the difference in complexation-induced changes in τ_c between MMP and α-cyclodextrin is even more dramatic than that reported in Table 4.

Taking the results obtained with α-cyclodextrin and palmitoyl-CoA as a model for complexation-induced restrictions in molecular mobility that occur *in the absence of a conformational change* in the polysaccharide, we conclude from the respective τ_c values that MMP must undergo a major conformational transition from a less to more ordered structure as it complexes acyl-CoA. This result is clearly consistent with a random coil→helix conformational change.

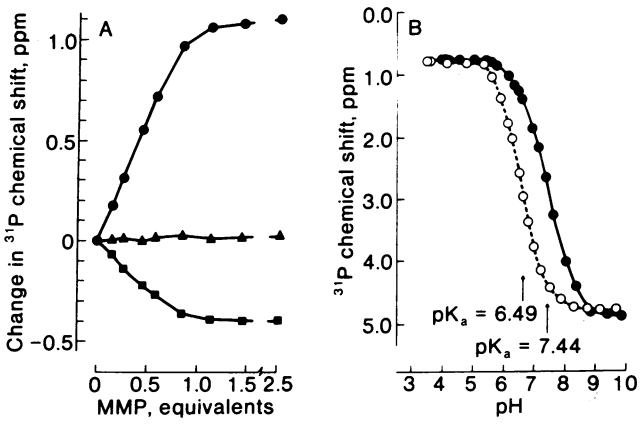

FIG. 3. (*A*) Changes in the ^{31}P chemical shifts of palmitoyl-CoA induced by complexation with MMP. ●, 3′-Phosphate; ■, α- and β-pyrophosphates; ▲, potassium phosphate buffer (control). (*B*) Measurement of the pK_a of the 3′-phosphate of palmitoyl-CoA in the presence (O---O) or absence (●—●) of MMP. The reported pK_a values are determined from the slopes of plots of ν_{HA} against $\nu_{HA}/[H^+]$. The pK_a of CoASH, in the presence or absence of MMP, is 6.37 (data not shown). All experiments were done in 1 mM phosphate/1 mM Na_2EDTA/99% ^2H_2O at 30°C. The pH was adjusted with ^2HCl or NaO^2H. Estimated error in the fitted pK_a values is ±0.05 pK_a unit.

Another important difference between α-cyclodextrin and MMP is seen in their respective kinetics of complexation with palmitoyl-CoA. Solutions of α-cyclodextrin with about 0.25 equivalent of CoASR display exchange broadening of the cyclodextrin ^{13}C signals at 68 MHz, resulting in a considerable increase in linewidths. No such broadening is observed with MMP. Kinetics of complexation of CoASR by α-cyclodextrin at 37°C are thus less than 1/10th as fast as the kinetics of complexation of CoASR by MMP. This difference in kinetics seems reasonable because the cyclodextrin complex can be formed only by "threading" the fatty acyl chain through the macrocycle, methyl end first. This restriction does not apply to the linear polysaccharide MMP.

Optical rotations and column chromatography (11, 12) and, more recently, fluorescence spectroscopy (27) have shown that MMP binds acyl-CoA derivatives, presumably the ligands in the physiological system, much more tightly than free fatty acids. ^1H, ^{13}C, and ^{31}P chemical shifts confirm the involvement of the CoA moiety in complexation of acyl-CoA with MMP (Table 5 and Fig. 3). There are significant differences between the chemical shifts of the CoA moiety in the acyl-CoA–MMP complex and in free CoASH, as well as in the complex and CoASR micelle.§ The data suggest that both the pantetheine and sugar phosphate moieties are involved in the complexation.

Further evidence for the involvement of the CoA moiety in complexation comes from direct comparisons of the MMP–

‡ MMP also interacts with, and probably encloses, guest molecules other than long-chain fatty acids and their CoA derivatives. Visible spectroscopy indicates that the polysaccharide forms a 1:1 complex with methyl orange (*p*-dimethylaminoazobenzenesulfonate) and fluorescence spectroscopy shows that 1-anilino-8-naphthalenesulfonate is also complexed by MMP. In the latter case, a hydrophobic MMP "pocket" is indicated by a characteristic increase in fluorescence and blue shift. The changes in the ^{13}C and ^1H NMR spectra of MMP induced by these guests are qualitatively similar to those induced by palmitoyl-CoA.

§ Two further controls confirm the conclusion. First, differences are also noted if phosphopantetheine and 3′,5′-ADP rather than CoASH are used for comparison, thus eliminating any folding in free CoASH (26) as an explanation for the discrepancies. Second, there are differences between the chemical shifts of the CoA moiety in the α-cyclodextrin–CoASR complex and the MMP–CoASR complex, consistent with the observation that the cyclodextrin does not show the same degree of binding preference as the mycobacterial polysaccharide for acyl-CoA derivatives over free fatty acids.

palmitic acid complex and the MMP–palmitoyl-CoA complex. A ^1H NMR study of the former has been reported recently (25). The protons of the polysaccharide show considerable similarity in the two complexes, although signal overlap prevents accurate comparisons of many resonances. Upon complexation by MMP, the α-CH_2 protons of free palmitic acid shift upfield and the β-CH_2 protons shift downfield, whereas the α-CH_2 protons of palmitoyl-CoA shift downfield and the β-CH_2 protons shift upfield. Other fatty acyl protons are shifted downfield in both cases. There are sizable (>0.5 ppm) differences in the ^{13}C chemical shifts of MMP in the two complexes (Table 1), notably at C-2 and C-6, predicted from the model (11) to be on the exterior of the MMP helix, and thus available for interaction with the CoA moiety. In contrast, differences in ^{13}C chemical shifts of α-cyclodextrin between the α-cyclodextrin–palmitoyl-CoA and α-cyclodextrin–palmitic acid complexes are negligible.

The apparent involvement of the CoA moiety in the MMP–acyl-CoA complex is also supported by chemical evidence. The thioester linkage in the MMP–acyl-CoA complex is 5 times more resistant to hydrolysis at pH 11.5 than it is in the α-cyclodextrin–acyl-CoA complex, suggesting more effective sequestering of this bond in the former case (unpublished results).

The fact that the major fatty acid methylene ^{13}C resonances of CoASR are shifted downfield by complexation with MMP is noteworthy because it is opposite to the direction expected for a micelle→monomer transition (41). Possibly, the fatty acyl chain is more fully extended in the complex than in the micelle; a decrease in *gauche* interactions found in the former would then account for the observed shift. [MMP does not bind linoleyl-CoA (5); presumably the kinked fatty acyl chain cannot be accommodated in the MMP channel.] Van der Waals interactions with the polysaccharide may also contribute to the downfield shift.

I am deeply indebted to Prof. K. Bloch for advice and support of this work. The electronic expertise of B. W. Bangerter and D. Ruben in modifying, respectively, the 100-MHz and 270-MHz instruments to allow inverse gated decoupling is gratefully acknowledged. I also thank A. Brown for technical assistance with the thioesterase assays and J. Simms for growth of *M. smegmatis* cells. The high-field NMR experiments were performed at the NMR Facility for Biomolecular Research located at the F. Bitter National Magnet Laboratory, Massachusetts Institute of Technology. The NMR Facility is supported by Grant RR00995 from the Division of Research Resources of the National Institutes of Health and by the National Science Foundation under Contract C-670. This work was supported by grants from the National Science Foundation, the National Institutes of Health, and the Eugene P. Higgins Fund of Harvard University. J.E.M. was a National Science Foundation Predoctoral Fellow 1976–1978.

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