Purification and Properties of the Fatty Acid Synthetase from Mycobacterium phlei

Dennis E. Vance, Osamu Mitsuhashi, and Konrad Bloch

From the James Bryant Conant Chemical Laboratories, Harvard University, Cambridge, Massachusetts 02138

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SUMMARY

The fatty acid synthetase from Mycobacterium phlei has been purified 340-fold to homogeneity. The enzyme has a molecular weight of \(1.39 \times 10^6\). At low concentrations of phosphate buffer (0.005 M), the synthetase dissociates into an enzymatically inactive species (7.65 S) which can be partially reaggregated and reactivated by dialysis against 0.5 M potassium phosphate buffer.

The mycobacterial polysaccharides, 3-O-methylmannose-containing polysaccharide (MMP) and 6-O-methylglucose-containing polysaccharide (MGLP), stimulate the fatty acid synthetase markedly. Their presence lowers the \(K_m\) values for acetyl-CoA and malonyl-CoA 4-fold and 4-fold, respectively. The polysaccharides also appear to function by altering the rate-limiting step of fatty acid synthesis. MMP stimulates fatty acid synthesis more effectively than GMLP. Various chemical modifications of the polysaccharides do not markedly alter their stimulating activity. Acetyl-CoA is the most effective primer and its concentration affects the degree to which MMP and GMLP stimulate fatty acid synthesis. It is proposed that the polysaccharides function primarily by binding long chain acyl-CoA and thereby relieve product inhibition of the fatty acid synthetase.

Multi-enzyme complexes catalyzing the synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA have been isolated from various eucaryotic sources and their properties are known in great detail (1). That such multi-enzyme complexes can occur also in prokaryotic organisms was demonstrated by the presence of a fatty acid synthetase of high molecular weight in Mycobacterium phlei (2). In most bacteria, fatty acid synthesis is catalyzed by individual, nonaggregating enzymes (8). Apart from comparative and phylogenetic aspects, the M. phlei fatty acid synthetase is of considerable interest because of its exceptional cofactor and substrate requirements, its relative instability, and the bimodal fatty acid pattern that this enzyme system produces (2, 4, 5). We now describe a procedure for purifying the My. phlei synthetase to homogeneity, conditions for dissociating and regenerating the complex, and the influence of substrate and cofactor concentrations on enzyme activity.

EXPERIMENTAL PROCEDURE

Materials—Acetyl-CoA was synthesized by the method of Simon and Shemin (9). [2-\(^{14}\)C]Malony-CoA was purchased from New England Nuclear. TPNH, DPNH, and FMN were obtained from Calbiochem. All other acyl-CoA derivatives and DTT were purchased from P. L. Biochemicals, Inc., DEAE-cellulose from the Brown Company, \(\alpha\)-amylase from Worthington Biochemicals, and Bio-Gel products from Bio-Rad Laboratories. Oyster glycogen, yeast mannan, and fat-free bovine serum albumin were purchased from Sigma, and DEAE-Sephadex was obtained from Pharmacia.

M. phlei ATCC-356 cells were grown to stationary phase (48 hours) on a medium containing glucose and Tween-80 (2).

Isolation of Polysaccharides—M. phlei cells (80 g) were suspended in 400 ml of distilled water and heated at 60°C for 15 min. This extract was fractionated as previously described up to and including Bio-Gel P-10 chromatography (4). The mixture of polysaccharides obtained from the Bio-Gel P-10 column was chromatographed on a DEAE-Sephadex column as described by Keller and Ballou (7). The MMP eluted with distilled water and the 6-O-methylglucose-containing polysaccharides (MGLP-I, -II, -III) were eluted with a 0 to 0.15 M NH\(_4\)HCO\(_3\) gradient. The separated polysaccharides were lyophilized and further purified by passage through a Bio-Gel P-6 column (3 x 75 cm) with distilled water as eluting solvent. After acid hydrolysis, carbohydrates were characterized and quantitatively identified by gas chromatography of the trimethylsilyl derivatives of the methyl glycosides as previously described (4). Polysaccharides were quantitatively estimated with a-naphthol reagent with D-glucose as a standard (8). The yield from 80 g of cells was 17 mg of MMP, 5 mg of MGLP-I, 14 mg of MGLP-II, and 16 mg of MGLP-III.

Fatty Acid Synthetase Assay—Fatty acid synthesis was measured by incorporation of [2-\(^{14}\)C]malonyl-CoA (2 \(\mu\)Ci per nmole) into fatty acids as previously described (2). The final concentrations of the components in the standard assay were 0.1 M potassium phosphate, pH 7.0; 5 mM DTT, 30 \(\mu\)M DPNH, 30 \(\mu\)M TPNH, 30 \(\mu\)M FAD, 0.25 mM ATP, 1 mM phosphoenolpyruvate, 0.5 mg of bovine liver phosphoenolpyruvate carboxykinase, 1 mg of fatty acid synthetase, and 0.2 \(\mu\)Ci [2-\(^{14}\)C]malonyl-CoA.

The abbreviations used are: DTT, dithiothreitol; MMP, 3-O-methylmannose-containing polysaccharide; MGLP, 6-O-methylglucose-containing polysaccharide; MGLP-I, MGLP-II, MGLP-III, 2 moles of succinyl residues per mole of MGLP; MGLP-I, MGLP-II, MGLP-III, 2 moles of succinyl residues per mole of MGLP; MGP, deacylated MGLP; BSA, bovine serum albumin.

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TPNH, and 1 μM FMN, and the final volume was 0.5 ml. Unless otherwise indicated, reaction mixtures contained 20 μM malonyl-CoA, 300 μM acetyl-CoA, 200 μM of crude polysaccharide (fraction after the Bio-Gel P-10 step), and 1 μg of enzyme. At low concentrations of enzyme (1 to 4 μg/0.5 ml), fatty acid synthesis was linear with time for at least 30 min, but all reactions were terminated after 15 min. One unit of activity is defined as the amount of enzyme required to incorporate 1 μ mole of malonate per min into the fatty acids.

In some instances the results obtained by radioactive assay were checked and confirmed by the spectrophotometric assay described by Lynen (9) with the above concentrations of substrates and cofactors.

Chemical Modification of Polysaccharides—For deacylation, a sample of MGLP-II (5 mg) was dissolved in 1 ml of 0.5 N NaOH and allowed to stand at room temperature for 1 hr. The product (MGP), after desalting by passage over a Bio-Gel P-6 column, contained less than 0.4 μ mole of acyl residue per mole of MGP as judged by a hydroxamate assay (10). MGLP-II contains 7 μ moles of acyl residues per mole of polysaccharide (7, 11).

MMP or MGPL was partially methylelated according to the method of Falvelon and Adams (12). Polysaccharide (1 mg) was dissolved in 0.5 ml of distilled water and 0.3 ml of dimethyl sulfate was added dropwise at 0°C, followed by 0.6 ml of 30% NaOH. The mixture was stirred overnight at room temperature and the additions of dimethyl sulfate and NaOH were repeated at this time and again after 8 hours. The reaction mixture was neutralized with 6 N HCl and the partially methylated polysaccharide was extracted 3 times with 4-ml portions of chloroform. Evaporation of the solvent yielded 0.8 mg of product. An 80-μg aliquot of the methylated polysaccharide was subjected to methanalysis and gas chromatography of the trimethylsilyl derivatives as previously described (4). MMP methylated by this procedure contained no glucose or 6-O CH3-glucose and methylated MMP contained neither mannose nor 3-O CH3-mannose, indicating that at least one additional methyl group had been introduced into each saccharide residue in the polysaccharide.

MGP was digested with α-amylase to remove 3 sugar residues from the nonreducing end of the polysaccharide as described by Snieker and Ballou (11). A 4.6-μg sample of MGP was dissolved in 1 ml of 0.09 M potassium phosphate, pH 7.0, containing 0.006 M NaCl. α-Amylase (1.75 mg) was added and, after 48 hours at room temperature, digestion was terminated by boiling the reaction mixture for 5 min; the precipitate was removed by centrifugation and discarded. The α-amylase-treated MGP was desalted by percolation through a Bio-Gel P-6 column (34 × 3 cm), lyophilized, and dissolved in 1 ml of distilled water (yield 2.42 mg). A 125-μg sample was subjected to methanalysis and the methyl glycosides were analyzed by gas chromatography of the trimethylsilyl derivatives (4). By comparison of the area of the 3-O CH3-glucose and the glucose peaks to that of 6-O-CH3-glucose peak obtained from MGP and α-amylase-digested MGP, it was calculated that α-amylase digestion had removed the 3-O-CH3-glucose residue and 2 glucose residues from the polysaccharide.

Purification of Fatty Acid Synthetase—All steps were performed at 0-4°C. Protein was determined by the method of Warburg and Christian (260 and 280 nm) (13). All buffers contained 1 mM DTT and 1 mM EDTA. Eighty grams of frozen M. phlei cells were thawed in 300 ml of 0.1 M potassium phosphate buffer, pH 7.0. The cells were broken by passage through a French pressure cell operated at 8,000 p.s.i., and the disrupted cells were centrifuged at 17,000 × g for 20 min. The resulting supernatant was centrifuged at 105,000 × g for 90 min and subsequently brought to 35% saturation with ammonium sulfate. After stirring for 15 min, the precipitate was removed by centrifugation at 37,000 × g for 20 min and discarded. The supernatant was slowly brought to 55% saturation, stirred for 30 min, and centrifuged at 37,000 × g. The 35 to 55% ammonium sulfate precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.0), and 32.8 ml of a 1% protamine sulfate solution (0.12 mg of protamine sulfate per mg of protein) were added dropwise while stirring. After 30 min the solution was centrifuged at 37,000 × g and the precipitate discarded. The supernatant was percolated onto a DEAE-cellulose column (3 × 20 cm) that had been previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. The column was washed with 1 liter of 0.25 M potassium phosphate buffer, pH 7.0. The enzyme was eluted by a linear gradient of 650 ml of 0.25 M potassium phosphate (pH 7.0) and 0.70 M potassium phosphate buffer (pH 7.0). The major fractions containing activity were concentrated to 50 ml on a Diaflo apparatus with an XM-50 membrane. The concentrated solution was slowly brought to 60% saturation with ammonium sulfate and centrifuged at 37,000 × g for 20 min. The precipitate was dissolved in 2 ml of 0.5 M potassium phosphate, pH 7.0, and applied to a Bio-Gel A-5m column (70 × 2.5 cm) that had been equilibrated with the same buffer. The enzymatic activity eluted with the first protein peak from the column. The collected peak fractions were combined and concentrated to 2.5 ml on a Diaflo apparatus with an XM-50 membrane. The results of this purification are summarized in Table I.

Physical Properties of Fatty Acid Synthetase—After 340-fold purification the enzyme was homogeneous as judged by centrifugation in a Beckman model E analytical ultracentrifuge (top of Fig. 1).

Sedimentation velocity studies of the purified enzyme in 0.5 M phosphate buffer gave an S value of 23.81. From this number, and using a Stokes radius of 108 A (10), a molecular weight of 1.39 × 106 was calculated (14). A partial specific volume of 0.725 cm3 per g was assumed. During dialysis for 24 hours at 4°C against 0.005 M potassium phosphate buffer, pH 7.0, containing 1 mM DTT and 1 mM EDTA, the enzyme complex dissociated into a smaller species with an S value of 7.65 (bottom of Fig. 1) corresponding to a molecular weight of 0.25 × 104.

As shown in Fig. 2, the enzyme rapidly loses fatty acid synthetase activity on dialysis in 0.005 M phosphate with a half-time of less than 1 hour. All activity had disappeared after 10 hours.

![Table I](image-url)

**Table I**

| Purification of fatty acid synthetase |
|-------------------------------------|
| **Total** | **Total protein** | **Specific activity** | **Yield** | **Purification** |
|----------|-------------------|----------------------|-----------|-----------------|
| units    | mg                | units/mg             | %         | fold            |
| Crude supernatant: | | | | |
| Ammonium sulfate | 3280 | 3466 | 1.10 | (100) | (1) |
| (35-55%) | 2739 | 1250 | 2.18 | 71.5 | 2.0 |
| Protamine sulfate | 3063 | 1231 | 2.49 | 80.0 | 2.3 |
| DEAE-cellulose | 1101 | 43.5 | 26.7 | 30.3 | 24.3 |
| Ammonium sulfate | 1109 | 30.5 | 36.4 | 29.0 | 33.1 |
| (0.6%) | | | | |
| Bio-Gel A-5m. | 656 | 1.75 | 375 | 17.1 | 341 |
FIG. 1. Sedimentation pattern of fatty acid synthetase complex (top) and dissociated enzyme (bottom) after 40 min at 37,020 rpm. Protein concentrations were 5 mg per ml in 0.5 M potassium phosphate buffer, pH 7.0 (top), and 5 mg per ml in 0.005 M potassium phosphate, pH 7.0 (bottom). Both buffers contained 1 mM DTT and 1 mM EDTA.

FIG. 2. Inactivation and reactivation of fatty acid synthetase by altering potassium phosphate concentration. Ten milligrams of enzyme in 1 ml of 0.5 M potassium phosphate buffer (pH 7.0) were dialyzed against 200 ml of 0.005 M potassium phosphate buffer, pH 7.0, for 20 hours at 4°C. At this time (indicated by the arrow) the dialysis buffer was changed to 0.5 M potassium phosphate and dialysis was continued for 12 hours. Aliquots of enzyme were removed at the times indicated and assayed for fatty acid synthetase activity as described under "Experimental Procedure." All buffers contained 1 mM DTT and 1 mM EDTA.

FIG. 3. Effect of acetyl-CoA concentration on fatty acid synthesis with 200 µg of PS (unresolved mixture of polysaccharides after Bio-Gel P-10 chromatography) (X--X) or without PS (O--O). The enzymatic assay was performed as described under "Experimental Procedure." The incubations without PS contained 140 µM malonyl-CoA and those with PS contained 80 µM malonyl-CoA.

Effects of Polysaccharides on Fatty Acid Synthesis—In an earlier report (4), we demonstrated that the polysaccharides lowered the apparent K_m for acetyl-CoA. At that time, TPNH (600 µM) was the only source of reductant used in the assay mixture. Later it was found that DPNH as well as TPNH is required for optimal fatty acid synthesis (5). Under these conditions, the rate of malonyl-CoA incorporation was about 2.5 greater than with 600 µM TPNH alone (5). For this reason, the kinetic effects of polysaccharide on fatty acid synthesis as a function of acetyl-CoA concentration were reinvestigated with assay mixtures containing 30 µM each of DPNH and TPNH.

In the presence of polysaccharide, acetyl-CoA saturates the synthetase at concentrations between 400 and 600 µM (Fig. 3); further increases in acetyl-CoA concentration cause inhibition. Without polysaccharide, acetyl-CoA does not reach saturating levels until about 2000 µM. There is less than a 2-fold difference in maximum velocities achieved with or without polysaccharide. However, the apparent K_m for acetyl-CoA is lowered by the polysaccharide from approximately 800 µM to 90 µM.*

2 The following difficulty arises in estimating K_m values for a two-substrate reaction in the presence or absence of a modifier as in the present instance. First of all, the concentration of one substrate (malonyl-CoA) affects the K_m value for the other (acetyl-CoA, (4)). Second, the optimal malonyl-CoA concentrations are 140 µM and 80 µM, respectively, in the absence and presence of polysaccharide, the former (140 µM) being slightly inhibitory when the assay is done in the presence of polysaccharide. Hence, we had two options: (a) to use the same malonyl-CoA concentrations
The assay was performed under standard conditions except that acetyl-CoA concentration in the incubations without PS was 2400 pM and in the incubations with PS was 600 pM.

FIG. 4. Effect of malonyl-CoA concentration on fatty acid synthesis with 200 μg of PS (X—X) or without PS (●—●). The assay was performed under standard conditions except that acetyl-CoA concentration in the incubations without PS was 2400 pM and in the incubations with PS was 600 pM.

FIG. 5. Fatty acid synthesis as a function of polysaccharide concentration. The standard assay was used except that the concentration of acetyl-CoA was 600 pM and that of malonyl-CoA was 100 μM. X—X, MMP; ○—○, MGLP-I; ■—■, MGLP-II; ●—●, MGLP-III.

Kₘ values for acetyl-CoA in the M. phlei system are unusually high compared to other fatty acid synthetase systems which generally operate with Kₘ values for acetyl-CoA of less than 50 μM (15).

Earlier we reported (4) that polysaccharide lowers the Kₘ for acetyl-CoA 50-fold, from 200 to 4 μM. These differences can be attributed to modification in assay conditions, i.e. substitution of 600 μM TPNH by 30 μM each of DPNH and TPNH.

The effect of polysaccharide on the apparent Kₘ for malonyl-CoA is less striking than for acetyl-CoA (Fig. 4). The value is reduced from about 40 μM to 9.6 μM.

Relative Effectiveness of Polysaccharides—The apparent Kₘ (concentration of polysaccharide at which one-half maximum stimulation is observed) for each of the polysaccharides was determined from a Lineweaver-Burk plot of the data shown in Fig. 5. In order to compare the effects of the various polysaccharides, fatty acid synthetase activity was plotted as a function of polysaccharide concentrations at saturating concentrations of acetyl-CoA and malonyl-CoA. The apparent Kₘ for MMP was estimated to be 8 μM (16.6 μg per ml); for MGLP-I, 23 μM (86 μg per ml); for MGLP-II, 34 μM (130 μg per ml); and for MGLP-III, 30 μM (116 μg per ml). Despite the marked structural differences between MMP (16) and MGLP (11), the magnitude of their effects on the rate of fatty acid synthesis under these conditions is not strikingly different. The various species of MGLP which differ only in their acyl content have closely similar activities but they are all inferior to MMP.

At suboptimal concentrations of acetyl-CoA, the differences in stimulatory effects of MMP and MGLP become greatly magnified (Fig. 6). Whereas at 300 μM acetyl-CoA stimulation by MGLP and MMP is nearly the same, at 20 μM acetyl-CoA MGLP is less effective than MMP by a factor of 8. In addition, acetyl-CoA appears to influence the apparent Kₘ for MMP. From a Lineweaver-Burk plot of the data in Fig. 6, it is calculated that at 300 μM acetyl-CoA the Kₘ for MMP is 20 μM and 1250 μM at 20 μM acetyl-CoA. Acetyl-CoA appears to modify the complex, altering its affinity for polysaccharide.

Effect of MGLP on MMP Stimulation—Fatty acid synthetase activity is roughly proportional to polysaccharide at low concentrations and the effects of MGLP and MMP are then additive (Fig. 7). But at saturating concentrations of MMP, stimulation is not potentiated by MGLP. If the two polysaccharides acted at different sites, a synergistic effect would be expected.

Effects of Polysaccharide Structure—Table II compares the effects of various natural and chemically modified polysaccharides on fatty acid synthesis. Partial methylation of MMP with incorporation of at least one additional methoxy group per hexose residue did not alter its stimulating activity. When MGLP was decarboxylated to MGP, stimulating activity declined only slightly. Furthermore, removal of the 3 carbohydrate residues from the nonreducing terminal of MGP by α-amylase did not diminish the effect on fatty acid synthesis. On the other hand, partial methylation of MGP did sharply lower the capacity to stimulate the synthetase. Glycogen, mannan, and...
FIG. 7. Combined effects of MGLP and MMP on fatty acid synthesis. The assay was performed as described under “Experimental Procedure” except that the acetyl-CoA concentration was 600 μM and malonyl-CoA was 100 μM. X—X, no MGLP-III; O—O, 50 μg of MGLP-III; •—•, 200 μg of MGLP-III.

**TABLE II**
Specificity of polysaccharide stimulation

Each of the polysaccharides was prepared and assayed for stimulating activity as described under “Experimental Procedure.” The observed stimulation is the fatty acid synthetase activity in the presence of polysaccharides, divided by the activity in the absence of polysaccharides.

| Polysaccharide/0.5 ml | Stimulation |
|-----------------------|-------------|
| MMP (100 μg)          | 10.3        |
| Methylated MMP (100 μg) | 10.3      |
| MGLP-II (100 μg)      | 6.1         |
| MGP (100 μg)          | 5.4         |
| α-Amylase-treated MGP (100 μg) | 5.4    |
| Methylated MGP (100 μg) | 1.8       |
| Oyster glycogen (300 μg) | 1.2      |
| Yeast mannan (300 μg)  | 1.3         |
| Bovine serum albumin (300 μg) | 0.5 |

bovine serum albumin were essentially inactive as stimulating agents.

While the structure of the native polysaccharides from M. phlei can be substantially modified without elimination of stimulating activity, the results with glycogen and mannan demonstrate that certain structural features, including perhaps the hydrophobic methoxy group, are essential.

**Primer Specificity of Fatty Acid Synthesis**—In view of the report by Lin and Kumar that butyryl-CoA is the preferred substrate for fatty acid synthesis in the mammary gland system (17), various short chain acyl-CoA derivatives were tested as primers for fatty acid synthesis. The results in Table III clearly demonstrate that acetyl-CoA is the most effective primer for the M. phlei fatty acid synthetase, yet the stimulation by the polysaccharide is greater for the higher homologues. In the range of 25 μM to 400 μM, butyryl-CoA and hexanoyl-CoA were essentially inactive unless polysaccharide was present. Since acetyl-CoA is the only primer that is significantly active in the absence of polysaccharide, a special activating effect of acetyl-CoA on the synthetase is again indicated.

**Effect of Polysaccharide on Rate-determining Step in Fatty Acid Synthesis**—In a study on an acetyl transferase, Riddle and Jencks (18) have demonstrated that “an observed Michaelis constant may reflect either a binding of substrate or a change in rate-determining step.” A plot of fatty acid synthetase activity against malonyl-CoA concentration in the presence or absence of MMP at 900 μM acetyl-CoA (Fig. 8) suggests that polysaccharide may function similarly, i.e. by changing the step in fatty acid synthesis which is rate-limiting. At concentrations of 4 μM or less, malonyl-CoA activity is virtually the same with or without MMP. It seems likely that at such low concentrations of malonyl-CoA, malonyl transacylase activity is rate-determining. As the malonyl-CoA concentration is raised, the rate of fatty acid synthesis without MMP levels off, indicating that some other reaction has become limiting. With MMP present in the incubation, the velocity increases almost linearly up to about 10 μM malonyl-CoA and above that concentration continues to rise with a much steeper slope than in the absence of MMP. Following the arguments of Riddle and Jencks, we may interpret these data to show that the apparent Kₐ for malonyl CoA without MMP (3.3 μM) reflects a change in the rate-determining step in fatty acid synthesis from malonyl transacylase to another reaction (18). When MMP is present, the apparent Kₐ (28.5 μM) may be equal to or closer to Kₐ, the true dissociation constant for malonyl-CoA. Curves of similar type were obtained at 300 μM and 600 μM.
acetyl-CoA, and in these instances also the $K_m$ values for malonyl-CoA differed in the presence and absence of MMP.

**Discussion**

The *M. phlei* fatty acid synthetase shares a number of properties with functionally analogous multienzyme complexes from other sources but displays certain features that are unique.

The molecular weight of this enzyme ($1.39 \times 10^6$) falls within the range observed for other fatty acid synthetase complexes (0.5 to 2.5 $\times 10^6$) (2). We had earlier reported a molecular weight of 1.7 $\times 10^6$ for the *M. phlei* synthetase, a value based on gel filtration and sucrose density gradient centrifugation of the enzyme in 0.1 M phosphate buffer (2). We consider the present value ($1.39 \times 10^6$) determined by sedimentation-velocity analysis of the synthetase in 0.5 M phosphate to be the more reliable.

Dissociation of the *M. phlei* enzyme on exposure to media of low ionic strength affords a subunit of the same molecular weight (0.25 $\times 10^6$) that has been observed for the fatty acid synthetases from animal tissues (19) and yeast (20). Dissociation and the consequent inactivation of the *M. phlei* synthetase are relatively rapid. The enzyme partially reaggregates to the enzymatically active form but only in buffers of relatively high ionic strength (0.5 M phosphate). It is of interest to note that dissociation and reassociation do not alter the characteristic specificity of the enzyme, i.e., the bimodal fatty acid product pattern or the relative rates of total synthesis from acetyl-CoA and palmitoyl-CoA elongation to $C_{36}$ and $C_{20}$ acids.

Comparison of the conditions affording optimal activity of fatty acid synthetases from various sources shows striking differences of which susceptibility to ionic strength is one. Fig. 9 shows the response to ionic strength of three fatty acid synthetase complexes under investigation in this laboratory. As noted, the synthetase from *M. phlei* is unstable and inactive in phosphate buffer below 0.1 M. A high molecular weight synthetase from *Euglena gracilis* is fully active in 0.1 M phosphate and rapidly inactivated as ionic strength is raised (21). Another bacterial multienzyme complex, isolated by H. Knoche from *Corynebacterium diphtheriae* (22), shows only slight activity in 0.1 M phosphate and is optimally active at 0.5 M phosphate. What is being observed is probably a combination of effects on catalytic activity and state of aggregation, but whatever their nature, it is clear that the physiological milieu in which the three enzyme systems operate in the cell must differ vastly.

One of the distinctive properties of the *M. phlei* synthetase is the unusually high $K_m$ for acetyl-CoA, a value about 20 times greater than reported for fatty acid synthetases from any other source. For comparative purposes we show corresponding values for the enzyme systems from other sources (Table IV). The very dramatic reduction of the $K_m$ values for acetyl-CoA in *M. phlei* by MMP and MGLP, with the consequence that they allow fatty acid synthesis to occur at more physiological substrate levels, has been reported previously (4). Polysaccharide also lowers the $K_m$ value for malonyl-CoA, but the reduction is of lesser magnitude. While we still lack a complete explanation for these effects, we wish to record here several observations and some speculations which bear on the mode of action of these polysaccharides.

Examining several of the partial reactions catalyzed by the fatty acid synthetase in order to localize the polysaccharide effect, we found none that was significantly stimulated by MMP or MGLP.* It was also observed that these partial reactions are strongly inhibited by palmitoyl-CoA. The inhibitions are relieved by inclusion of BSA or of MMP or MGLP in the reaction mixtures. The BSA-like effects of the polysaccharides suggested that they might bind or complex palmitoyl-CoA and thereby lower the effective concentration of this potent enzyme inhibitor. Such interactions between the mycobacterial polysaccharides and palmitoyl-CoA have, in fact, been demonstrated by chromatography on Sephadex (23). If palmitoyl-CoA, which is one of the products of fatty acid synthesis, inhibited the system either by a generalized detergent effect or by negative feedback, then its removal due to sequestering with polysaccharides could result in an acceleration of over-all synthesis. Yet, as previously reported (2), BSA will not replace polysaccharide as a stimulatory agent for over-all fatty acid synthesis by the *M. phlei* system. This observation suggests that binding of palmitoyl-CoA or other long chain end products is not the only mechanism by which polysaccharides influence fatty acid synthesis in the *M. phlei* system.

An accompanying paper describes the effects of MMP and MGLP on the fatty acid synthetases from yeast and *C. diphtheriae* (23). In these systems polysaccharide stimulation is significant but much less marked than in *M. phlei*. Moreover, in these two systems, in contrast to *M. phlei*, the polysaccharide effects are fully reproducible by BSA. It would, therefore, appear that the mycobacterial polysaccharides play a species-specific role in

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*O. Mitsuhashi, unpublished observations.

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TABLE IV

| Source               | Acetyl-CoA $K_m$ | Malonyl-CoA $K_m$ |
|----------------------|-----------------|-------------------|
| Yeast$^a$            | 28.0            | 8.0               |
| Rat liver$^b$        | 4.4             |                   |
| Pigeon liver$^b$     | 2.1             | 8.0               |
| Mammary gland$^c$    | 22              | 13.0              |
| *Corynebacterium diphtheriae* | 25           | 10.0              |
| *Mycobacterium phlei* | 800 (90)$^e$   | 40.0 (10)$^e$     |
| *Euglena gracilis* (etiolated) | 3.5           | 45.5              |

$^a$ Data taken from Lynen (9).

$^b$ Data taken from Smith and Abraham (15).

$^c$ In presence of polysaccharide.

$^e$ T. Esders and P. Flick, unpublished observations.
regulating fatty acid synthesis in *M. phlei*. If the polysaccharides function in *M. phlei* also by binding long chain acyl-CoA and, therefore, relieving inhibition of fatty acid synthesis from acyl-CoA, the question arises as to why HSA will replace the ridges function in *S. phlei* also by binding long chain acyl-CoA exposed on the enzyme surface, a large molecule such as bovine serum albumin (mol wt 69,000) might be unable to approach it, whereas this site might be more accessible to a smaller molecule (MMP, mol wt 2,100). As a result, polysaccharide and long chain acyl-CoA could interact to form a relatively hydrophilic complex which would more readily diffuse into the aqueous environment. On the other hand, to explain the equivalence of BSA and polysaccharide in their effects on the yeast and *C. diptheriae* system (23), one need only assume that in these instances the end product (palmitoyl-CoA) diffuses from the enzyme at an appreciable rate. The complexing agents can then bind free palmitoyl-CoA and thereby protect against end product inhibition.

The above scheme for the mechanism of polysaccharide stimulation of the *M. phlei* fatty acid synthetase accounts for many of the experimental findings reported in this paper. For example, if the size of the binding molecule (polysaccharide or BSA) were important for access to the enzyme site and hence for interaction with long chain acyl-CoA, and if the two polysaccharides were to bind the acyl-CoA derivatives with the same affinity, then one would expect MMP (mol wt 2100) to be more effective than MGLP (mol wt 3700) for stimulating fatty acid synthesis. MMP does, indeed, have a $K_a$ value of $3$ to $4$ times lower than MGLP.

It should be pointed out that the comparative effectiveness of MMP and MGLP is a function of acetyl-CoA concentration. The two polysaccharides stimulate fatty acid synthesis equally at high acetyl-CoA concentrations (300 $\mu$M), whereas at 70 $\mu$M acetyl-CoA, MMP is superior to MGLP by a factor of 8. One can rationalize these results by postulating that high acetyl-CoA concentrations induce a conformational change which makes the enzyme complex equally accessible to the two polysaccharides of different size.

To account for the lowering of the $K_m$ for acetyl-CoA by polysaccharide, the following explanation is offered. If acetyl CoA and C$_{14}$-CoA compete for the same enzyme site and if this site has a greater affinity for C$_{14}$-CoA, then the apparent $K_m$ for acetyl-CoA will be a function of the C$_{14}$-CoA concentration. Polysaccharides, by binding the C$_{14}$-CoA, will, therefore, lower the apparent $K_m$ for acetyl-CoA.

The bioplastic chain length pattern of the end products, apart from posing special problems of regulation, makes it highly probable that the *M. phlei* synthetase serves multiple purposes. For example, it might provide fatty acids not only for membrane phospholipids but also for the complex lipids of the mycobacterial cell envelope. The membrane phospholipids of *M. phlei* contain mainly C$_{16}$ and C$_{18}$ acids while the C$_{22}$ and C$_{24}$ acids are probably building blocks for the class of wall components known as mycolic acids (24). Devices are, therefore, needed for controlling the supply of shorter and longer chain synthetase products for the two separate biosynthetic pathways and it is attractive to implicate the mycobacterial polysaccharides in this regulatory role.

Whether or not this speculation is correct, one further aspect of fatty acid synthesis in *M. phlei* is puzzling. *M. phlei* contains, apart from the multienzyme complex under discussion (type I), a second fatty acid synthetase (type II) which is acyl carrier protein-dependent and functions strictly as an elongating system, converting palmitoyl-CoA or stearoyl-CoA to C$_{22}$ and C$_{24}$ products (2, 25). Since the type I synthetase also produces these long chain acids, either by total synthesis or by elongating palmitoyl-CoA, the type II synthetase would appear to be redundant. Another possibility is that the acyl carrier protein-dependent elongating system arises artifically from the multienzyme complex during the processing of the bacterial extracts. However, the experimental evidence so far does not favor this explanation. Throughout purification of Synthetase I, the relative activities for total synthesis and palmitoyl-CoA elongation remain constant and no conditions have so far been found for either inactivating the complex differentially or dissociating it into functionally separate units.

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