

Fatty Acid Synthetase Activity in *Mycobacterium phlei*: Regulation by Polysaccharides

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ABSTRACT The multienzyme complex from *Mycobacterium phlei* which catalyzes the synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA requires a heat-stable fraction (stimulating factor, SF) for activity. Fractionation of heat-treated *M. phlei* extracts affords two stimulatory subfractions, one of which (SF₂) can be replaced by FMN. The other (SF₁) is further separable into 3 polysaccharides (PS_I, PS_{II}, and PS_{III}). PS_I contains about 95% 3-O-methylmannose and 5% mannose; the sugar composition of PS_{II} and PS_{III} is about 55% 6-O-methylglucose and 45% glucose for both. Each of the three purified polysaccharides, in combination with FMN, substitutes for the crude stimulating factor. The polysaccharides exert their effect on the fatty acid synthetase by lowering the K_m for acetyl-CoA about 50-fold.

In *M. phlei*, fatty acid synthesis from acyl-CoA and malonyl-CoA is catalyzed by two apparently independent enzyme systems (1). One of these is a multienzyme complex of the type found in yeast (2) and animal tissues (3) (Type I) while the other (Type II) resembles the nonaggregated acyl carrier protein (ACP)-dependent system occurring in *Escherichia coli* and other bacteria (4-6). Noteworthy features of the *M. phlei* Type I synthetase are (1): (1) The various enzymes that constitute elongating activity behave as a single physical entity with a molecular weight of 1.7×10^6 . (2) The multienzyme complex is unstable in solutions of low ionic strength. Below 0.1 M phosphate, synthetase activity declines rapidly ($t_{1/2} = 1$ min at 4°C in 0.01 M phosphate buffer) with disaggregation of the complex to lower molecular weight fragments. (3) At low acetyl-CoA concentrations the formation of fatty acids is almost totally dependent on a heat-stable fraction obtainable from *M. phlei* extracts.

The uncharacterized *M. phlei* factor (1) has now been shown to owe its activity to two components. One of these is replaceable by FMN. The other is a mixture of three polysaccharides.

EXPERIMENTAL

The *M. phlei* Type I fatty acid synthetase was the 30-fold purified preparation already described (1). A synthetase preparation obtained by a modification of our earlier procedure (H. B. White, unpublished) had a specific activity 150 times that of crude extract. Enzyme activity was determined by measuring simultaneously the incorporation of ³H from [³H]acetyl-CoA and of ¹⁴C from [¹⁴C] malonyl-CoA into pen-

tane-soluble fatty acids. The reaction components and their concentrations and assay conditions were identical with those used earlier (1). For assay of stimulating factor activity, the concentration of acetyl-CoA was 20 μM. After a flavin had been identified as one of the active components of SF, FMN (0.8 μM) was routinely added to reaction mixtures.

Isolation of SF₁ and SF₂

Crude SF was either prepared as described earlier (1) or more conveniently as follows. A suspension of 100 g of water-washed *M. phlei* cells in 500 ml of distilled water was boiled with stirring for 30 min. After cooling, the supernatant was separated from the coagulated cells by centrifugation at 8000 rpm for 15 min. The extract was concentrated by lyophilization to a small volume and an equal volume of acetone was added. The resulting precipitate was removed by filtration and discarded. Acetone was added to the filtrate to a final concentration of 90%, and the precipitated material was collected by centrifugation and dried by washing with ether. The resulting dry powder was redissolved in 2.5 ml of water and chromatographed on a 42 × 2.5 cm column of Bio Gel P-10 with water or 0.1 M phosphate buffer, pH 7, as the eluent (see Fig. 1). Column fractions were assayed. Partial SF activity was recovered in eluent fractions 48-74 (SF₁). Full activity was obtained by combination of SF₁ with eluant fractions 110-120 (SF₂). The yellow SF₂ fractions, which stimulated synthetase activity only in the presence of SF₁, contained both FAD and FMN as shown by paper chromatography in *n*-butanol-(acetic acid)-water 4:1:5 (upper phase).

The early Bio Gel P-10 peak (SF₁, Fig. 1) gave strongly positive tests for sugar. Further purification, and the elution pattern of SF₁ during subsequent chromatography, was therefore monitored with α-naphthol reagent by measuring absorbances at 555 nm (7).

Acetylation

A sample of SF₁ containing 1 mg of sugar (glucose equivalent) was acetylated in 1 ml of acetic anhydride-pyridine at 65°C for 30 min. The mixture was evaporated to dryness, and the residue extracted with chloroform. The acetylated, chloroform-soluble product contained 0.66 mg of sugar (glucose equiv.) and had 20% of the specific activity of SF₁ in the fatty acid synthetase assay. Deacetylation by heating in anhydrous-ammoniacal methanol for 30 min at 50°C restored the original specific activity.

Abbreviations: SF, stimulating factor; PS, polysaccharide (stimulatory).

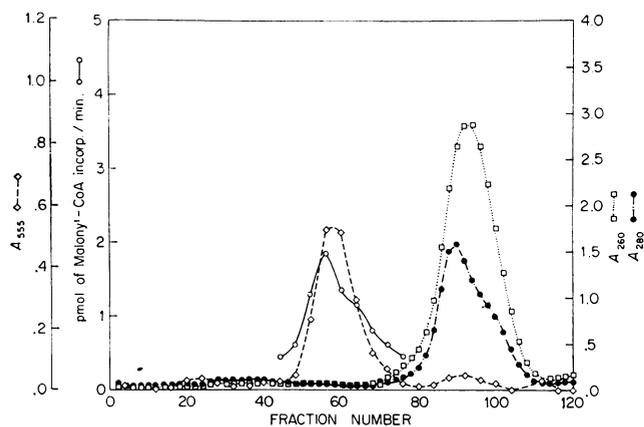


FIG. 1. Bio Gel P-10 chromatography of *M. phlei* stimulating factors. Aqueous extract (2.5 ml), corresponding to 100 g of cells, was carried through the 50–90% acetone precipitation step, applied to a Bio Gel P-10 column, 100–200 mesh (42×2.5 cm), and eluted at 4°C with phosphate buffer, pH 7.0. The collected fractions (2.6 ml each) were analyzed for carbohydrate with α -naphthol reagent (A at 555 nm) and for protein and nucleic acid by measuring A at 280 and 260 nm, respectively. For assaying stimulation of *M. phlei* fatty acid synthetase by column fractions, incubation mixtures contained, in a total volume of 0.5 ml, 20 μ M [14 C]malonyl-CoA, 20 μ M [3 H]acetyl-CoA, 300 μ M TPNH, 0.8 μ M FMN, 1 mM dithiothreitol, 0.1 M phosphate buffer (pH 7.0), and 2.7 μ g of enzyme protein. Samples were incubated at 37°C for 1 hr.

Separation of polysaccharides

An aqueous solution of SF₁ was chromatographed on a Bio Gel P-6 column with distilled water as the eluting solvent. Three α -naphthol-positive fractions emerged (PS_I, PS_{II}, and PS_{III}). Stimulating activity, as measured in the standard assay supplemented with FMN, was associated with all three fractions. The elution patterns for absorbance at 555 nm and stimulatory activity coincided closely (see Fig. 2).

Fraction PS_I was further purified by passage through the cation-exchanger AG 50W-X8 followed by chromatography on Bio Gel P-6. This treatment reduces the protein content of the fraction to less than 1% relative to sugar. Amino acids, amino sugars, or other ninhydrin-positive materials could not be detected in PS_I in the amino acid analyzer after hydrolysis in 6 N HCl for 24 hr. PS_I had a specific rotation of $[\alpha]_D = +83^\circ$ ($c = 0.039$) in H₂O.

Carbohydrate composition of polysaccharides

PS_I. Samples were converted into their methyl glycosides by heating in 0.75 N HCl–methanol for 16 hr at 65°C. The methanolysis products were converted to the trimethylsilyl [(CH₃)₃Si] derivatives according to Sweeley, *et al.* (8) and analyzed by gas-liquid chromatography on a 6-ft column of 3% OV-1. The temperature was programmed at 5°C per min from 140 to 230°C. The chromatogram showed two peaks. One of these comprised 6% of the total area and had the same retention time as methyl [tetra-*O*-(CH₃)₃Si] mannopyranoside (7.4 min.). The major peak (94% of the total area, retention time 5.4 min.) was characterized as follows: (1) mass spectrometry of the (CH₃)₃Si methyl glycoside mixture gave a fragmentation pattern with two major ions at *m/e* 146 and *m/e* 217. A molecular ion was not observed, but an ion at

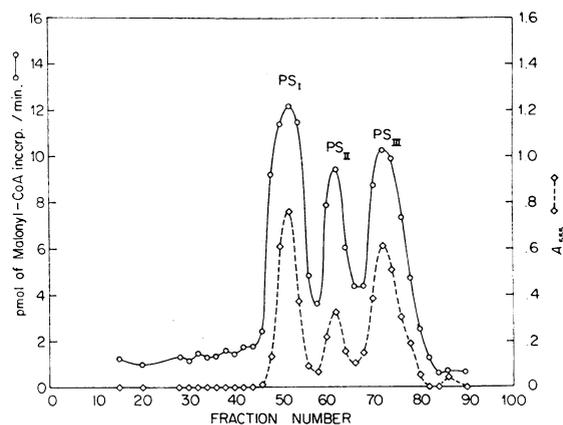


FIG. 2. Bio Gel P-6 chromatography of polysaccharide mixture. A solution of SF₁ (fractions 48–74, Fig. 1), containing 30 mg of carbohydrate (glucose equiv.), was applied to a column of Bio Gel P-6, 100–200 mesh (70×2.5 cm) and eluted with distilled water at 4°C. Fractions (3.7 ml each) were assayed for carbohydrate with α -naphthol reagent and for stimulatory activity for fatty acid synthetase as described in the legend to Fig. 1.

m/e 378 (loss of OCH₃ and CH₃) indicated an *O*-methylhexose (9). (2) Treatment of the mixed methyl glycosides with 0.1 M NaIO₄ (10) failed to destroy the *O*-methylhexose. After reaction with periodate, the product still formed the (CH₃)₃Si derivative with a retention time of 5.4 min. This experiment proves substitution of the C₃ position. (3) Permethylation of the methyl glycosides with dimethyl sulfate (11) afforded a product that was identical with methyl (tetra-*O*-methyl) mannopyranoside but separated from the corresponding glucose or galactose derivatives on gas-chromatography. The major sugar of PS_I is, therefore, identified as 3-*O*-methylmannose. This sugar has been previously isolated from a bacterial source by Candy and Baddiley (12).

PS_{II} and PS_{III}. These fractions were methanolized and the resulting methyl glycosides converted into the (CH₃)₃Si derivatives as described for PS_I. The gas-liquid chromatograms of the monosaccharide derivatives derived from PS_{II} and PS_{III} were identical. Both showed a peak with the retention time of 6-*O*-methylglucose (8.8 min) and peaks for α -glucose (9.8 min) and β -glucose (10.2 min). Bound acyl groups were determined according to Lee (13).

RESULTS AND DISCUSSION

Properties of stimulating factor (SF)

Conditions chosen for assaying the ability of SF to enhance fatty acid synthesis were saturating concentrations of malonyl-CoA (20 μ M), TPNH (300 μ M)*, and limiting concentrations (20 μ M) of acetyl-CoA. In later experiments, the assay system also contained 0.8 μ M FMN. Under these conditions fatty acid synthesis was linear with time. The molar ratio of malonyl units to acetyl units incorporated ranged from 9 to 11.

* Recently, it has been found that a mixture of 30 μ M DPNH and 30 μ M TPNH increases fatty acid synthetase activity 2 to 3-fold over that obtained with 300 μ M TPNH as the sole source of pyridine nucleotide. Under these conditions the addition of saturating amounts of polysaccharide raises acetyl-CoA and malonyl-CoA incorporation 50 to 100-fold.

TABLE 1. Properties of polysaccharide fractions

Fraction	Carbohydrate composition, %*				Acyl content†	Specific activity‡
	Mannose	3-O-Methylmannose	Glucose	6-O-Methylglucose		
PS _I	4.5 (7.4)†	95.5 (5.4)	0	0	0	250
PS _{II}	0	0	54.0 (9.8, 10.2)	46.0 (8.8)	7-9	153
PS _{III}	0	0	56.5	43.5	9-12	97

* Areas of each peak on the gas chromatogram, uncorrected for possible differences in the detector response.

† Bracketed numbers are retention times (min) of (CH₃)₃Si methyl glycosides after acid-catalyzed methanolysis. The retention times given for glucose are those of the α -glucose and β -glucose derivatives, respectively.

‡ Moles of fatty acid, as determined by hydroxamate assay, per mole of polysaccharide. The molecular weights of PS_{II} and PS_{III} are assumed to be 3-4000, on the basis of the identity of their sugar composition with that of the lipopolysaccharide described by Saier and Ballou (14).

§ Specific activity is defined as the ratio of stimulated to unstimulated fatty acid synthetase activity under standard assay conditions.

Chromatography of the previously described factor (1) on Bio Gel P-10 yielded an active fraction (SF₁) that emerged immediately after the void volume (Fig. 1). However, even in saturating concentrations, this fraction was only about half as active as crude SF. The more strongly retained column fractions when tested alone failed to stimulate the synthetase. A combination of one of the later fractions (SF₂, elution volume in the region of a vitamin B₁₂ marker) with optimal amounts of SF₁ showed full stimulating activity. Since SF₂ fractions were yellow, the presence of flavin nucleotides was suspected. FAD or FMN, indeed, replaced SF₂ in the synthetase assay. The presence of FAD and FMN in SF₂ fractions was confirmed by paper chromatography. At optimal concentrations, FMN was 2-3 times as active as FAD.

Initial tests revealed the following properties of SF₁. Pronase, trypsin, RNase, DNase, α -amylase, β -amylase, dextranase, emulsin, and alkaline phosphatase failed to diminish the biological activity of the factor. Precipitability by ethanol or acetone, and insolubility in chloroform or ether, ruled out a conventional lipid. The gel filtration behavior on Bio Gel P-10 and the slow diffusion of the material on prolonged dialysis suggested medium-sized polymeric molecules. All active fractions contained sugar. Treatment with periodate under conditions known to cleave vicinal glycols partially inactivated SF₁; acid conditions leading to the hydrolysis of glycosidic bonds destroyed stimulating activity. SF₁ was, therefore, assumed to be a polysaccharide. In support of this diagnosis, acetylation of SF₁ gave chloroform-soluble product that stimulated fatty acid synthesis only slightly but regained full activity on mild saponification. Also, sodium borate, a complexing agent for carbohydrates, was inhibitory.

Fractionation on Bio Gel P-6 separated SF₁ into three sugar-containing fractions with coincident biological activities (Fig. 2). On the basis of sugar content (glucose equiv.), the relative specific activities of these fractions (PS_I, PS_{II}, and PS_{III}) in stimulating fatty acid synthesis were 2.5:1.5:1 (Table 1).

Characterization of polysaccharides

Fraction PS_I contains 3-O-methylmannose as the major, and mannose as a minor, component (Table 1). The molecule is neutral and contains no reducing ends. No other sugars were detectable on gas chromatography of the trimethylsilyl derivatives of the methyl glycosides after acid-catalyzed metha-

lysis of PS_I. The material appears to be closely related to a *M. phlei* polysaccharide isolated independently by Gray and Ballou.† The specific activities of the two polysaccharides in the fatty acid synthetase assay were found to be equal.

Fractions PS_{II} and PS_{III} both contain 6-O-methylglucose and glucose as the only sugars and in about the same ratio (55:45). Their stimulatory activities are also similar but somewhat less than that of PS_I (Table 1). A lipopolysaccharide of identical sugar composition and containing various fatty acids in ester linkage has been described by Saier and Ballou (14). A deacylated sample of this lipopolysaccharide showed much weaker synthetase-stimulating activity than either PS_{II} or PS_{III}. Whether the differences in chromatographic behavior between PS_{II} and PS_{III}, and the lower biological activity of the deacylated lipopolysaccharide of Saier and Ballou, are related to the content of esterified acyl group in the same basic polysaccharide structure is now being investigated.

Mode of action of stimulating factors

The following observations clarify, to some extent, the mode of action of the heat-stable factors in the synthesis of fatty acids by the *M. phlei* multienzyme complex. Under ordinary assay conditions; i.e., in the presence of malonyl-CoA, TPNH, and low (20 μ M) concentrations of acetyl-CoA, the synthetase shows weak and variable activity. This low activity can be raised many-fold either by supplementing with stimulating factor (SF) or by substantially increasing the concentration of acetyl-CoA. Raising the acetyl-CoA concentration from 20 to 200 μ M enhances synthetase activity up to 10-fold, but the maximal stimulation at saturation is only about 30% of the optimal activity obtained with saturating amounts of SF and 20 μ M acetyl-CoA (1). After FMN was identified as one of the components of SF, the relationship between acetyl-CoA concentration and synthetase activity was reinvestigated. When assay systems were supplemented with FMN, activity again depended markedly on acetyl-CoA concentrations, but incorporation was several times greater in the presence than in the absence of the nucleotide (Fig. 3). Thus, in a system saturated with respect to acetyl-CoA, FMN becomes rate limiting for the over-all process of fatty acid synthesis.

† Private communication: G. Gray and C. E. Ballou. Their polysaccharide also contains 3-O-methylmannose and mannose, and a total of 18-20 sugar units.

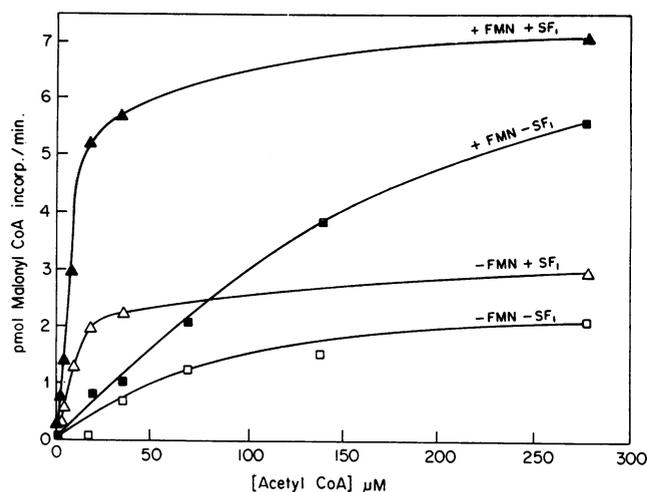


FIG. 3. Effects of FMN and polysaccharide (SF_1) on the fatty acid synthetase of *M. phlei*. Assay conditions as in the legend to Fig. 1. When indicated, $0.8 \mu\text{M}$ FMN and/or $43 \mu\text{g}$ of polysaccharide (SF_1) was added to the reaction mixtures.

At low concentrations of acetyl-CoA ($20 \mu\text{M}$), the synthetase is stimulated up to 30-fold by crude SF_1 (1). Under these conditions of limiting substrate concentration, the separate and combined effects of FMN and purified polysaccharide (SF_1 or PS_I , PS_{II} , and PS_{III} individually) are as follows (Fig. 3): The addition of FMN alone raises the synthetase activity slightly. When only polysaccharide (SF_1) is added, activity rises to the extent obtained with saturating concentrations of acetyl-CoA in the absence of both SF_1 and FMN. Finally, fatty acid synthesis is at a maximum, i.e., increases approximately another 3 times, on addition of both SF_1 and FMN. This rate is essentially identical to that with $200 \mu\text{M}$ acetyl-CoA in the presence of FMN, but without SF_1 . Thus, the synthetase can operate with the same efficiency under two sets of conditions: either at low and limiting substrate concentrations ($20 \mu\text{M}$ acetyl-CoA) in the presence of polysaccharide, or with high acetyl-CoA concentrations in its absence. The superimposed increase due to FMN is the same in both situations. While the stimulating polysaccharides effect synthetase activity only in the lower range of acetyl-CoA concentrations, no such dependence exists for the FMN requirement. Under all conditions tested, the nucleotide is rate limiting and stimulates the purified synthetase about 3 times.

Thus far, FMN has been shown to be present and to function as a prosthetic group (for enoyl reductase) only in the fatty acid synthetase of yeast (2). In this instance the nucleotide is very tightly bound, whereas it appears to dissociate, at least partially, during purification of the *M. phlei* synthetase. It remains to be demonstrated that FMN is also involved in the enoyl reduction in the bacterial enzyme system.

The nature of the polysaccharide effect on the synthetase system becomes clearer from a double reciprocal plot of the data. Fig. 4 shows the velocity of the synthetase reaction as a function of acetyl-CoA concentration at different concentrations of polysaccharide. V_{max} is independent of the concentration of the stimulating factor; the variable parameter is the K_m for acetyl-CoA. From the data in Fig. 4, it is calculated that in the absence of polysaccharide, the K_m for acetyl-CoA is $200 \mu\text{M}$; saturating amounts of polysaccharide lower it to

$4 \mu\text{M}$. In comparable experiments, the K_m of the synthetase for malonyl-CoA was found to be $1\text{--}2 \mu\text{M}$, either in the presence or absence of polysaccharide.

In the multienzyme system under study, a positive modifier affects the K_m for acetyl-CoA in the overall process. A logical site for this control is the initial transfer of acyl groups from acyl-CoA to a protein component of the complex. We have tested three early reactions in which the initial transacylase step could be rate-limiting and conceivably be under polysaccharide control. These are, the acyl-CoA:pantetheine transacylase reaction (15), the acyl-CoA-dependent CO_2 -malonyl-CoA exchange, and the synthesis of triacetic acid lactone. None of these reactions responded sufficiently to polysaccharide to account for the stimulation observed in the complete synthetase system. In view of these failures to demonstrate enhancement of an isolated step in fatty acid synthesis, the phenomenon of polysaccharide stimulation can be described only in general terms. These molecules markedly lower the K_m for acyl-CoA, the substrate that initiates and primes the elongation process. The result is to enable fatty acid synthesis to proceed at low, and thus more physiological, substrate concentrations.

Regulation of fatty acid biosynthesis by carbohydrate is an attractive mode of metabolic control as a possible alternative to the modulation of acetyl-CoA carboxylase. The activity of the latter enzyme is rate-limiting for fatty acid synthesis in animal tissues (16) but there is no evidence that the same is true in bacteria.

Since some of the *M. phlei* polysaccharides are "lipopolysaccharides," in the sense that they are esterified with various fatty acids (14), it is possible that their enhancing effects on

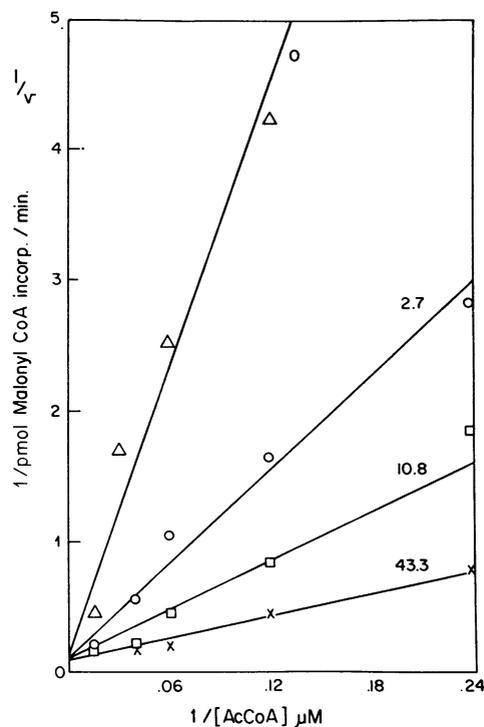


FIG. 4. The effect of polysaccharide (SF_1) on the K_m for acetyl-CoA with the *M. phlei* fatty acid synthetase. Assay conditions described in the legend to Fig. 1. The numbers on each line are the μg of SF_1 added to assay mixtures.

fatty acid synthesis are related to their ability to serve as acylacceptors.

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