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Effects of 6-*O*-Methylglucose-Containing Lipopolysaccharides on the Activity of Fatty Acid Elongation Systems in *Mycobacterium Smegmatis*

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ABSTRACT. Among four species of 6-*O*-methylglucose-containing lipopolysaccharides (MGLP)[1,2] of *Mycobacterium smegmatis*, MGLP-I, which lacks succinyl residues, and MGLP-II, which contains one mol of succinyl residue per mol of MGLP, inhibited the activity of the acyl carrier protein (ACP)-dependent fatty acid elongation system (FAS-II)[3] prepared from the same bacilli by ammonium sulfate precipitations and gel filtration of Sephacryl S-200. When the molar ratio of palmitoyl-CoA to MGLP-II was 1:1, the FAS-II activity was reduced to about 70% of the normal value. When it was 1:3, the activity was about 30%. MGLP-I, on the other hand, inhibited the activity of partially purified palmitoyl-CoA-ACP transacylase, which is one of enzymes of FAS-II. In addition, it also inhibited the activity of the acetyl-CoA-dependent fatty acid elongation system (FES-I)[4] when the concentration of decanoyl-CoA used as the optimum primer was lower than its 2 Km value and the activity of the malonyl-CoA-dependent fatty acid elongation system (FES-II)[5] when the concentration of stearoyl-CoA used as optimum primer was lower than 50 μM (the standard concentration of the original assay). The physiological role of these lipopolysaccharides on the mycobacterial lipid metabolism was discussed.

ABBREVIATIONS: MMP, 3-*O*-metylmhannose-containing polysaccharide; MGLP, 6-*O*-methylglucose-containing lipopolysaccharide; MGLP-I, MGLP with no succinyl residues; MGLP-II, 1 mol of succinyl residue per mol of MGLP; ACP, acyl carrier protein; FAS-I, fatty acid synthetase complex; FAS-II, ACP-dependent fatty acid elongation system; FES-I, acetyl-CoA-dependent fatty acid elongation system; FES-II, malonyl-CoA-dependent fatty acid elongation system; DTT, dithiothreitol; EDTA, ethylenediamine tetracetate

Key words : inhibition — 6-*O*-methylglucose-containing lipopolysaccharides — fatty acid elongation systems — palmitoyl-CoA-ACP-transacylase

The 3-*O*-methylmannose-containing polysaccharide (MMP) and 6-*O*-methylglucose-containing lipopolysaccharide (MGLP) of *Mycobacterium smegmatis* are well known to markedly stimulate the activity of the fatty acid synthetase complex (FAS-I) of this bacilli.[1] On the other hand, it has been reported that

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MMP had little effect on the activity of the ACP-dependent fatty acid elongation system (FAS-II) of *M. smegmatis*.[3]

However, MGLP-I, one of the MGLPs containing no succinyl residues and MGLP-II, containing one mol of succinyl residue per mol as reported by Gray and Ballou,[2] were found to inhibit the activity of FAS-II under a somewhat different condition than previously reported.[3] It was also found that MGLP-I inhibited the activity of palmitoyl-CoA-ACP transacylase, one of the enzymes of FAS-II. Furthermore, MGLP-I also inhibited the activity of the acetyl-CoA-dependent fatty acid elongation system (FES-I)[4] and the malonyl-CoA-dependent fatty acid elongation system (FES-II)[5] of *M. smegmatis* under certain conditions. These results are described in this paper.

MATERIALS AND METHODS

1) *Cultivation of M. smegmatis.* The strain of *M. smegmatis* used and methods for cultivation were the same as reported previously.[6] The bacilli were harvested at the early stationary phase (48 hrs after inoculation), washed thoroughly with water and then stored at below $-80°C$.

2) *Preparation of acyl carrier protein (ACP).* ACP was isolated from *M. smegmatis* by the method of Matsumura *et al.*[7] and further purified by polyacrylamide disc gel electrophoresis. The purity of ACP appeared to be greater than 96% from densitometric measurements.

3) *Preparation of MGLP.* MGLP was isolated from *M. smegmatis* by the hot water extraction-method[1] (Method I) or 70% ethanol extraction with reflux.[2] When ethanol extraction was used, the bacilli were first incubated in the presence of L-[methyl-^{3}H] methionine and then extracted with 70% ethanol. This extract was mixed with the 70% ethanol extract of non-labeled bacilli, and then MGLP was purified from this mixture according to the method[2] (Method II-a). MGLP was also isolated from the 70% ethanol extract of non-labeled bacilli directly. In this case, purification of MGLP was performed by isolating fractions which stimulate the activity of the fatty acid synthetase complex (FAS-I) of *M. smegmatis* in each purification step (Method II-b). MGLPs isolated by these three methods were hydrolyzed with 1 N HCl for 4 hrs at 100°C, after which the hydrolyzates were analyzed by cellulose thin layer chromatography (Avicel®-SF from Funakosi Pharmaceutical Co., LTD.) with solvent system, 1-butanol-pyridine-water (10 : 3 : 3, V/V). Glucose (Rf=0.18) and 6-*O*-methylglucose (Rf=0.36) were detected with an aniline hydrogen phthalate reagent.[8]

4) *Preparation of the ACP-dependent fatty acid elongation system (FAS-II).* FAS-II was isolated from *M. smegmatis* by a slight modification of the procedure of Odriozola *et al.*[3] The harvested cells (about 10 g wet weight) were washed with 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM DTT (buffer A) and then suspended in a four times volume of buffer A. Next, they were sonicated with Branson Sonifier Model 250 (20 KHz, 100 watts for 5 min), after which the sonicate was centrifuged at 20,000 × g for 20 min. The supernatant obtained was subjected to $(NH_4)_2SO_4$-fractionation to obtain a 55-90% saturated fraction. This fraction was dissolved in a small volume of buffer A and then dialyzed against buffer A for 3 hrs. The dialyzed solution was applied to a column of Sephacryl S-200 (2.0×46.5 cm) equilibrated with buffer A and eluted with the same buffer. Fig. 1 shows that the FAS-II

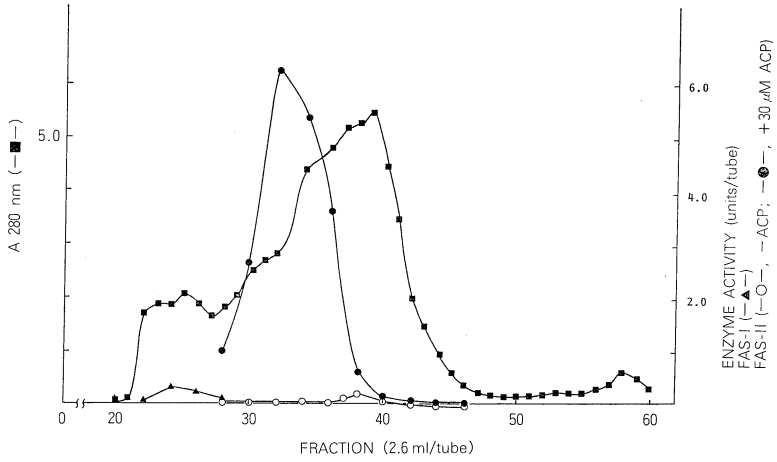

Fig. 1. Gel-filtration of the fatty acid synthetase complex (FAS-I) and the ACP-dependent fatty acid elongation system (FAS-II) of *M. smegmatis*. A 55–90% $(NH_4)_2SO_4$ fraction was applied to a Sephacryl S-200 column (2.0×46.5 cm) equilibrated with buffer A (described in the text) and eluted with the same buffer. One unit of activity is defined as the amount of enzyme required to incorporate 1 nmol of malonate per min into the fatty acids.

and FAS-I activities were partially separated. Furthermore, the FAS-I was weakened by using 0.1 M potassium phosphate buffer instead of 0.25 M (optimum for the FAS-I). Fractions from Nos 30 to 33 containing FAS-II activity were combined and concentrated with a membrane filter (Amicon Diaflo® Ultrafiltration Membranes PM-10), The concentrated solution was then divided into small tubes and stored at below −20°C. This preparation showed a strict dependency on ACP and proportionalities to the enzyme up to 51 μg protein and to the incubation time up to 30 min. The Km value for palmitoyl-CoA was about 20 μM.

5) *FAS-II assay*. This enzyme system was assayed by a partially modified procedure of Odriozola *et al.*[3] The reaction mixture containing 10 μmol of potassium phosphate buffer (pH 7.2), 10 nmol of NADH, 10 nmol of NADPH, 0.5 μmol of DTT, 10 nmol of ACP from *E. coli*, 4 nmol of [2-^{14}C]malonyl-CoA (14.1 MBq/mmol), various amounts of acyl-CoA and various amounts of MGLP for a total volume of 92 μl was preincubated at 37°C for 15 min. After addition of 8 μl of enzyme solution (28.6 μg protein) to the mixture, it was incubated at 37°C for 15 min. The reaction was stopped by adding 0.4 ml of water and 0.15 ml of 50% KOH. Then the mixture was saponified at 95°C for 30 min and extracted with 2 ml of n-pentane 3 times after acidification with 6 N HCl. The radioactivity in the dried extract was counted with a liquid scintillation counter.

6) *Purification of palmitoyl-CoA-ACP transacylase*. The transacylase was isolated from *M. smegmatis* by a slight modification of the procedure of Kervabon *et al.*[9] To remove palmitoyl-CoA thioesterase activity in the early step of purification, 55-90% $(NH_4)_2SO_4$ precipitate of a 105,000 × g supernatant was obtained. Since the purity achieved by the last procedure with DEAE-cellulose was not high enough, the transacylase was further purified by gel filtration through a Sephadex G-100 column. Polyacrylamide disc gel electrophoresis of

this preparation showed two bands and the purity was assumed to be more than 50% by densitometry, as described below. The specific activity of the preparation assayed with 100 μM ACP of *E. coli* showed about 144-fold purification of this enzyme.

7) *Palmitoyl-CoA-ACP transacylase assay.* The activity of palmitoyl-CoA-ACP transacylase was assayed by measuring the product of [1-^{14}C]palmitoyl-ACP, which was separated from [1-^{14}C]palmitoyl-CoA by addition of chloroform-methanol (1 : 2, V/V), as described by Mancha[10] The reaction mixture containing 20 μmol of imidazole-HCl buffer (pH 6.2), 1.0 μmol of DTT, 1.0 μmol of EDTA, 2.4 nmol ACP from *M. smegmatis*, 10 nmol of [1-^{14}C]palmitoyl-CoA (56.6 KBq/mmol), various amounts of MGLP and 34 μl of enzyme solution (0.58 μg protein) for a total volume of 0.2 ml was incubated at 37°C for 15 min. The reaction was stopped by adding 1 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 10 μmol $MgCl_2$ and 2 mg bovine serum albumin. One ml isopropanol, 25 μl acetic acid and 50 μl ammonium sulfate-saturated aqueous solution were added successively. The [1-^{14}C]palmitoyl-ACP and protein were coprecipitated by addition of 8 ml chloroform-methanol (1 : 2, V/V), and the precipitate was washed with 5 ml chloroform-methanol (1 : 2, V/V) six times. The washed precipitate was dried by heating at 50°C for 30 min and then dissolved in 4 ml of water. The radioactivity of [1-^{14}C] palmitoyl-ACP was counted by addition of 6 ml of instagel to the solution.

8) *Preparation of the acetyl-CoA-dependent (FES-I)[4] and malonyl-CoA-dependent (FES-II)[5] fatty acid elongation systems.* FES-I & FES-II were prepared by the method of Kikuchi and Kusaka.[5]

9) *Assay of FES-I & FES-II.* Assay of FES-I was performed by the method of Shimakata *et al.*[4] The tentative Km value for decanoyl-CoA in this system was about 37.5 μM. Since this system was not inhibited by MGLP-I with 90 μM decanoyl-CoA as primer, 6.8 μM and 60 μM decanoyl-CoA were used in this elongating reaction.

Assay of the FES-II system was performed by the method of Kikuchi and Kusaka.[5] In this assay 15μM stearoyl-CoA was used, because the activity was not inhibited by MGLP-I with 50 μM stearoyl-CoA (the standard amount of the original assay).

10) *Fatty acid synthetase complex assay.* This assay was carried out by the procedure of Vance *et al.*[1]

11) *Other procedures.* Carbohydrates were quantified with phenol-sulfuric acid reagent using D-glucose as the standard.[11,12] Proteins were determined by the method of Lowry *et al.* using bovine serum albumin as the standard.[13] As the microquantification, a total protein-estimating reagent, Tonein® -TP was used. Polyacrylamide disc gel electrophoresis of protein was carried out by the method of Davis.[14] The density of the protein-band was measured by absorbance at 560 nm after staining with Coomassie Brillant Blue R-250.

12) *Chemicals.* [2-^{14}C]malonyl-CoA (1.76 GBq/mmol), [1-^{14}C]palmitoyl-CoA (2.22 GBq/mmol), L-[methyl-^{3}H]methionine (>3.77 GBq/mmol), and β-[3-^{3}H(N)]-Alanine (4.4 TBq/mmol) were purchased from New England Nuclear, Boston. [1-^{14}C]acetyl-CoA (2.15 GBq/mmol) was obtained from the Radiochemical Centre, Amersham. Malonyl-CoA, acetyl-CoA, stearoyl-CoA were purchased from P. L. Biochemicals Inc. Palmitoyl-CoA was obtained from the Pharmacia LKB Biotechnology Group. ACP from *E. coli* (about 57% purity)

was purchased from Sigma Chemical Co. Tonein® -TP was obtained from Otsuka Pharmaceutical Co., Ltd., Otsuka Assay Laboratories. 6-*O*-methylglucose was synthesized from D-glucose by the procedure of Bell.[15] All other chemicals were reagent grade from commercial sources.

RESULTS

1) *Effect of MGLP on FAS-II activity.* When 20 μM palmitoyl-CoA was used as primer, the FAS-II activity was inhibited by MGLP-II, as shown in Fig. 2. When the molar ratio of palmitoyl-CoA to MGLP-II was 1 : 1, inhibition of the FAS-II activity was about 34%. When the ratio was 1 : 2, the inhibition was about 66%. When 50 μM palmitoyl-CoA was used as primer, the activity was also inhibited by MGLP-II, as shown in Fig. 2. When the molar ratio of palmitoyl-CoA to MGLP-II was 1 : 1 and 1 : 3, the inhibition was about 33% and 68%, respectively. These results suggest that inhibition of

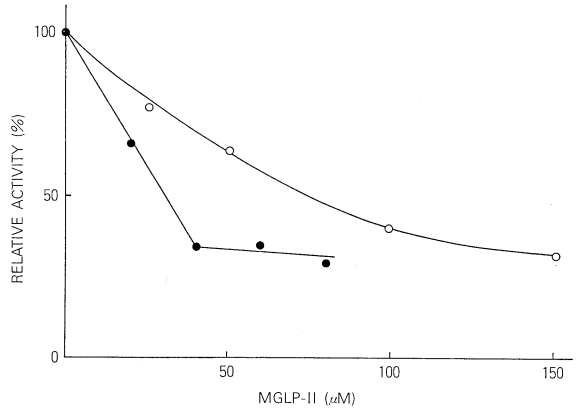

Fig. 2. Effect of MGLP-II on the FAS-II activity.
The assay condition was as described in MATERIALS AND METHODS with 20 μM palmitoyl-CoA (-●-) and 50 μM palmitoyl-CoA (-○-) as primer. MGLP-II was prepared by Method II-b, as described in MATERIALS AND METHODS.

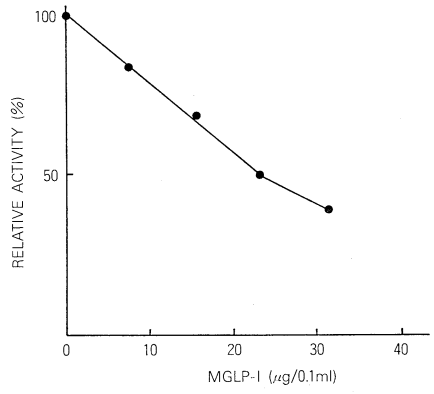

Fig. 3. Effect of MGLP-I on FAS-II activity.
The assay condition was as described in MATERIALS AND METHODS with 20 μM palmitoyl-CoA as primer. MGLP-I was prepared by Method I, as described in MATERIALS AND METHODS.

the FAS-II activity was affected by the molar ratio of palmitoyl-CoA to MGLP-II. The activity was less inhibited by MGLP-II with 50 μM stearoyl-CoA than with 50 μM palmitoyl-CoA (data not shown). The FAS-II activity was also inhibited by MGLP-I, as shown in Fig. 3. The inhibition was about 60% by about 30 μg MGLP-I.

The effect of MGLP-II on the FAS-II activity described above was resumed, as shown in Table 1.

2) *Effect of MGLP-I on the activity of palmitoyl-CoA-ACP transacylase.* The partially purified transacylase showed the maximum velocity with 50 μM

TABLE 1. Effect of MGLP-II on the activity of FAS-II

Palmitoyl-CoA concentration (μM)	Stearoy-CoA concentration (μM)	MGLP-II concentration (μM)	Specific activity of FAS-II (nmol/min/mg)	Inhibition (%)
20		0	1.17	—
20		20	0.77	34
20		40	0.40	66
20		60	0.40	66
50		0	2.82	—
50		25	2.23	21
50		50	1.88	33
50		100	1.13	60
50		150	0.91	68
	50	0	2.20	—
	50	25	2.09	5
	50	50	1.62	26
	50	100	1.10	50
	50	150	0.77	65

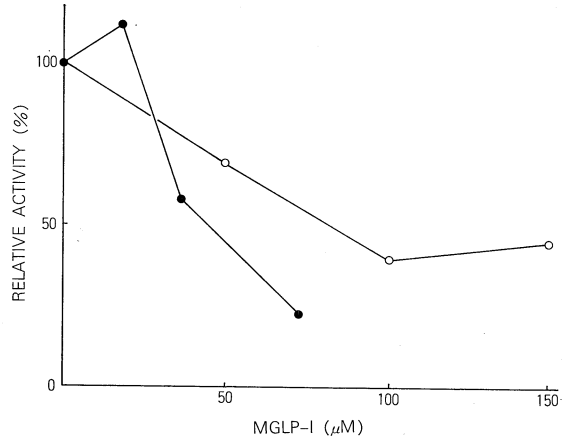

Fig. 4. Effect of MGLP-I on activities of palmitoyl-CoA-ACP transacylase and the malonyl-CoA-dependent fatty acid elongation system (FES-II). The assay conditions were as described in MATERIALS AND METHODS. -○-, transacylase; -●-, FES-II. MGLP-I was prepared by Method II-b (-○-) and Method II-a (-●-), as described in MATERIALS AND METHODS.

palmitoyl-CoA as primer. MGLP-I inhibited the activity with 50 μM palmitoyl-CoA, as shown in Fig. 4. When the molar ratio of palmitoyl-CoA to MGLP-I was 1 : 2, the inhibition was about 60%.

3) *Effect of MGLP-I on the activity of the fatty acid elongation system II.* The effect of MGLP-I on the activity of FES-II with 15 μM stearoyl-CoA is also shown in Fig. 4. When the concentration of MGLP-I was 36 μM, the inhibition was about 42%.

4) *Effect of MGLP-I on the activity of the fatty acid elongation system I.* MGLP-I inhibited the activity of FES-I, as shown in Fig. 5. The Km of this system for decanoyl-CoA was 37.5 μM. MGLP-I inhibited the activity more with 6.8 μM decanoyl-CoA than with 60 μM decanoyl-CoA.

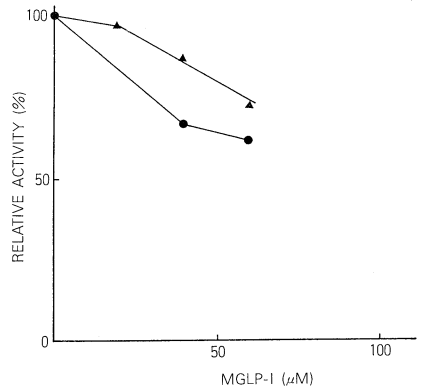

Fig. 5. Effect of MGLP-I on the activity of the acetyl-CoA-dependent fatty acid elongation system (FES-I).
The assay condition was as described in MATERIALS AND METHODS with 6.8 μM decanoyl-CoA (-●-) and 60 μM decanoyl-CoA (-▲-) as primer. MGLP-I was prepared by Method II-a, as described in MATERIALS AND METHODS.

DISCUSSION

As already mentioned, it has been reported that MMP had little effect on the activity of FAS-II of *M. smegmatis*.[3] However, the author found that MGLP-I and II did inhibit FAS-II activity under certain conditions, as described above. O-DI-methyl-β-cyclodextrin, which is known to stimulate FAS-I activity and to form complexes with palmitoyl-CoA and palmitic acid,[16] also inhibited FAS-II activity like MGLP-II (data not shown).

Yabusaki and Ballou have described that the intracellular concentration of long chain acylcoenzyme A in mycobacterial cells was about 0.3 mM and the concentration of the polymethylpolysaccharides approached 1 mM.[17] As already described, FAS-II activity was inhibited about 70% by MGLP-II when 150 μM MGLP-II and 50 μM palmitoyl-CoA were used. Under certain physiological conditions, FAS-II activity therefore seems to be regulated by polymethylpolysaccharides in mycobacterial cells.

Yabusaki and Ballou assumed the existence of a complex formation of palmitoyl-CoA with MGP for the thioesterase inhibition by MGP.[17] Machida and Bloch have also reported that MGLP-II forms a complex with palmitoyl-CoA at a ratio of 0.7 mol of palmitoyl-CoA to 1 mol of MGLP-II.[18] Based

on these reports, the inhibition of FAS-II activity by MGLP-I and II seems to be due to complex formation of palmitoyl-CoA with MGLP-I and II. Furthermore, the inhibition of FAS-II activity by MGLP-I and II seems to be related partially at least to inhibition of palmitoyl-CoA-ACP transacylase, which is one of enzymes in the FAS-II system.

Since the activities of FES-I and FES-II were also inhibited by MGLP-I, these fatty acid elongating systems seem to also be regulated by MGLP etc.

As described above, *de novo* fatty acid synthetase activity is stimulated by MGLP, which inhibits several kinds of fatty acid elongating activities. MGLP seems therefore to regulate *de novo* fatty acid synthesis and fatty acid elongation in opposite directions. MMP may also regulate fatty acid synthesis and elongation to form a complex with palmitoyl-CoA[18] like MGLP-II. This mechanism may be specific for *Mycobacterium* and may be involved in the regulation mechanism of mycolic acid-biosynthesis.

(Correction) The so-called fatty acid-binding protein of *M. smegmatis* reported previously by us[19] was found to contain some MGLP-fraction, which was assumed to be a real main fatty acid-binding element. Therefore, we must apologize for hoping to withdraw the previous report,[19] hereafter.

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