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## Brief Note

# Immunochemical Characterization of the Fatty Acid Elongation System II of *Mycobacterium smegmatis*

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**Key words : fatty acid synthesis — *Mycobacterium smegmatis* — acyl carrier protein — immunoblotting**

The general mechanism of *de novo* fatty acid synthesis and fatty acid elongation is similar to each other in a view of the sequential reactions: condensing of acyl derivatives with the appropriate units to form $\beta$-oxo-compounds, reduction of which to form secondary alcohols, then dehydration of these alcohols to *trans*-$\alpha,\beta$-double bonds and the final reduction of the double bonds. *Mycobacterium smegmatis* seems to be evolutionally or metabolically luxurious organism in respect to fatty acid synthesis, since this organism has two kinds of fatty acid synthetases (FAS; FAS-I and FAS-II)[1–5] and also two kinds of fatty acid elongation systems (FES; FES-I and FES-II).[6–9] FAS-I is rather animal type's multienzyme complex undertaking *de novo* fatty acid synthesis,[1,3,5] while FAS-II is a botanical type's non-aggregated system in which the component enzymes, assumed to be seven in number, jointly elongate a long chain acyl-CoA using malonyl-CoA as the condensing unit.[2,4] Both FAS-I and FAS-II require protein-bound 4'-phosphopantetheine (acyl carrier protein, ACP) during elongation.[3,4] In contrast with these FAS's, both FES-I (the acetyl-CoA dependent medium chain elongation system[6]) and FES-II (the malonyl-CoA dependent long-chain elongation sysyem[7–9]) are non-aggregated and ACP-nonrequiring systems. The existence of two kinds of non-aggregated malonyl-CoA dependent elongation systems in *M. smegmatis*, *i.e.* one is the ACP-requiring-FAS-II and the other is the ACP-nonrequiring-FES-II, appears to be surplus and rather puzzling. Accordingly, our attention was focused on ascertaining furthermore whether FES-II preparations truly contain ACP or not. The electrophoretic transfer of proteins to nitrocellulose and their subsequent localization with highly specific antibodies and $^{125}$I-labeled protein A (immunoblotting) has recently gained wide use to identifying biologically significant proteins[10–12] and unequivocally detecting picogram amounts of antigens of interest within cell extracts. This technique was applied, therefore, to identify ACP in the preparations.

ACP was purified according to Bloch *et al.*[2] A female rabbit (weighing approx. 2.5 kg) was immunized with intradermal injections on multiple site on the back. The immunogen consisted of 1.0 ml of solution of the purified ACP (1.5 mg/ml) emulsified with an equal volume of Freund's adjuvant. Complete adjuvant was used for the first, third, and fifth injection; incomplete Freund's adjuvant was used for the intervening injections. The rabbit received six immunizations at intervals of 7–10 days. Electrophoresis was carried out at 20 mA per slab gel at room temperature for 1 hr using Miniproteian-8 Apparatus (Bio-Rad). Fifteen percents polyacrylamide gel containing 0.1% SDS was

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employed. Proteins were transferred from SDS-slab-gel onto nitrocellulose paper according to Burnette[10] in a Trans-Blot Cell Apparatus (Bio-Rad) filled with buffer consisting of 25 mM tris, 192 mM glycine and 20% (v/v) methanol. Electroblotting was carried out at 400 mA for 2.5 hrs at 20°C. Following electrophoretic transfer, the nitrocellulose replica was rinsed with 50 mM Tris, pH 8.0, containing 150 mM NaCl (TBS), blocked for 30 min at room temperature with 5% BSA in TBS (B-TBS) and then incubated for 1 hr at room temperature with B-TBS containing the antiserum against *M. smegmatis* ACP diluted 1:100 in B-TBS. The blot was then washed with several change of TBS and incubated for 1 hr at room temperature with B-TBS containing $5 \times 10^5$ dpm/ml of $^{125}$I-labeled protein A (spc. act. 30 $\mu$Ci/mg, Dupont), followed by washing with TBS and air dry. Autoradiograms were obtained by exposing the nitrocellulose paper to AIF film (RX-L) for 24 hrs with enhancing screen. FAS-II and FES-II were prepared as reported previously.[7] The activities of FAS-II and FES-II were assayed by measuring the radioactivities incorporated from 2-$^{14}$C-malonyl-CoA to pentane-extractable products as reported previously.[7]

Antiserum raised in rabbit against *M. smegmatis* ACP inhibited FAS-II activity almost completely with 1 $\mu$l of that, while FES-II was not inhibited at all even with 5 $\mu$l of the antiserum (Fig. 1). Furthermore, in our experiments, *E. coli* ACP (Sigma) at a concentration of 40 mM had no effect on the incorporation of radioactivities from 2-$^{14}$C-malonyl-CoA into products by FES-II (data not shown). The FAS-II and FES-II preparations were subjected to electrophoresis, immunoblotting with anti-ACP serum. As shown in Fig. 2, only one band was seen autoradiographically in each lane 4 and 6 which corresponding to ACP, but not seen at all in lane 5. By these results, FES-II became clearly distinguishable from FAS-II, that is, FES-II does neither contain ACP nor require a supplement of ACP for the activity, while FAS-II absolutely requires the external supplement of ACP.

Taking account of the fact as mentioned above, a question arises how the mycobacterial cell does need physiologically FES-II for the synthesis of fatty acids also producible by FAS-II. This apparent redundancy could be partially

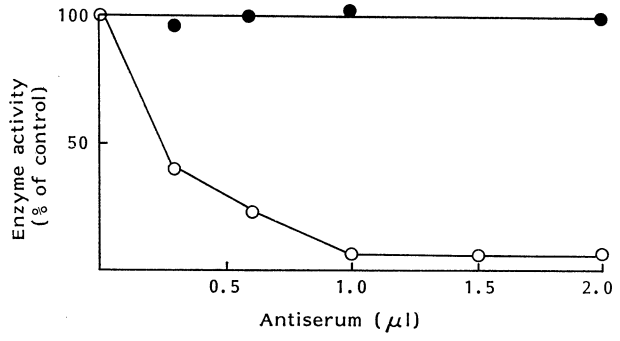


Fig. 1. Effect of the antiserum against *M. smegmatis* ACP on the activity of FAS-II or FES-II. FAS-II (●) or FES-II (○) was preincubated for 30 min at 30°C with increasing concentration of anti-ACP serum. The 100% value of FAS-II or FES-II activity was 2864 dpm/mg protein of 2-$^{14}$C-malonyl-CoA incorporation or 2690 dpm/mg protein of that incorporation into pentane-extractable products, respectively.

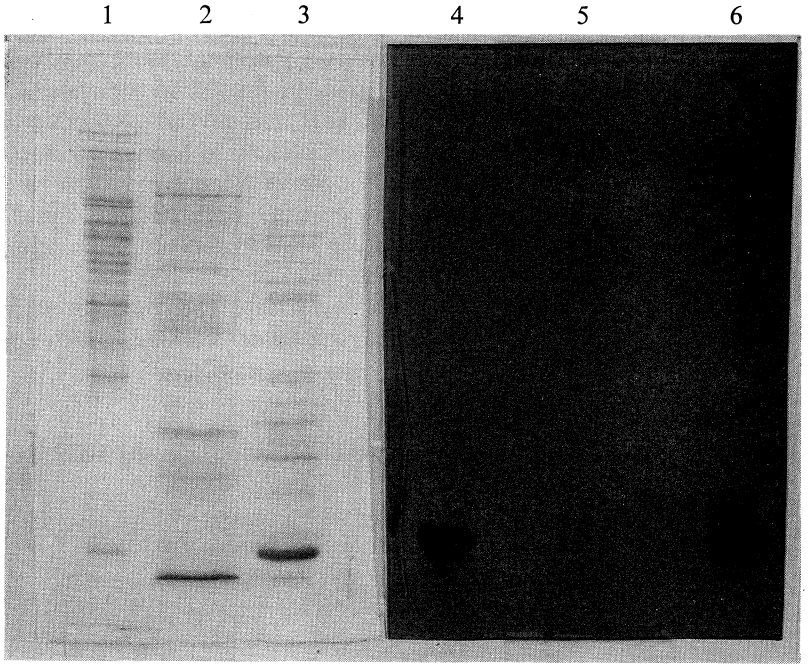


Fig. 2. Immunoblotting of FAS-II and FES-II from *M. smegmatis*. After electrophoresis of crude cell extracts (lane 1 and 4), FES-II (lane 2 and 5) and FAS-II (lane 3 and 6), proteins were stained with Coomassie Brilliant Blue (lane 1, 2 and 3) or transferred onto nitrocellulose paper. The paper was incubated with anti-ACP serum followed by incubation with $^{125}$I-labeled protein A. The dried paper was processed for autoradiography (lane 4, 5 and 6).

TABLE 1. Different features of *M. smegmatis* FAS-II and FES-II

| | FAS-II[4] | FES-II |
|---|---|---|
| Dependency on ACP | + | − |
| Requirement of pyridine-nucleotides | NADH or NADPH | NADH and NADPH[7] |
| Elongation products* | acyl-ACP derivatives (67%)<br>free acids (31%) | acyl-CoA derivatives (86%)<br>acyl-ACP derivatives (6.9%)<br>free acids (6.3%) |

*The nature of the elongation products was determined by the butanol fractionation procedure reported by Bloch *et al.*[4]

explained at least by a comparison of some features about the two enzyme systems (Table 1). For example, the choice of using of FAS-II or FES-II to supply C24 and longer-chain-fatty acids may depend on an intracellular ratio of NADH/NADPH or ACP content. If the levels of the reducing cofactors rise and those of ACP are low, the intracellular conditions may become more favorable for the activity of FES-II than that of FAS-II. Furthermore, as shown in Table 1, more than 86% of the products by FES-II were found as acyl-CoA derivatives, and only 6.3% and 6.9% were free acids and acyl-protein

derivatives, respectively. While about 67% of the products by FAS-II were free acids and 31% were acyl-ACP derivatives.[4] These facts might support, in some extent, the necessity of dual long chain-fatty acid elongation systems in *M. smegmatis* for further elongation of acyl derivatives. Recently, we have demonstrated a lignoceroyl-CoA desaturation system to form $\Delta^{15}$-tetracosenoate in the cytosol fraction of *M. smegmatis*.[13] Since lignoceroyl-CoA is one of the main products of FES-II, it may be possible that FES-II and this desaturation system act sequentially to form $\Delta^{15}$-tetracosenoate from long chain-fatty acids (~C18) though direct evidence has not obtained yet.

It might therefore be of interest to investigate the relative activities of FAS-II and FES-II in *M. smegmatis* harvested under various growth conditions such as the growth phase, temperature, carbon source, *etc.*

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