Fatty Acid Synthesizing Enzyme Activity of Cultured Mycobacterium lepraemurium

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For more than half a century, Mycobacterium lepraemurium has been well-known as an incultivable mycobacterium as has Mycobacterium leprae. Recently, it became possible, however, to cultivate M. lepraemurium by means of Ogawa’s method (14). This method presents an invaluable facility for biochemical study of this microorganism, because isolation and collection of its pure and native cells from the cultivation medium are evidently much easier than these procedures from host tissues. Owing to this cultivation, studies on fatty acid biosynthesis of M. lepraemurium, which has been almost unknown up to the present, can be carried out and it has been found that this microorganism contains quite a unique pathway for the synthesis of fatty acids as compared with Mycobacterium smegmatis, one of the rapid growing mycobacteria. These findings are presented in this paper.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The Hawaiian strain of M. lepraemurium and the ATCC 14468 strain of M. smegmatis were kindly supplied by Dr. T. Mori and Dr. K. Shoji of the Research Institute for Microbial Diseases, Osaka University, Osaka. A colony mass of M. lepraemurium was transplanted on 1% Ogawa’s yolk medium (14), supplemented with hemin as reported by Mori (14). After six weeks cultivation at 35°C, the cells grown in vitro were collected into centrifuge tubes and then washed thoroughly with water by centrifugation. M. smegmatis was grown on the surface of Sauton medium at 35°C for five days, then harvested and washed well with water.

Preparation of crude extracts. The cells of M. lepraemurium and M. smegmatis, respectively, were ground in a mortar for 45 minutes at 4°C with the same weight of quartz sand as wet weight of bacilli, then suspended in ten volumes of 0.1 M phosphate buffer (pH 7.2). The suspension was centrifuged at 10,000 x g for 40 minutes and the supernatant thus obtained was used as a crude extract of each mycobacterium. Contents of protein in these extracts were measured by the method of Lowry et al (18). Enzyme assays. Acetyl CoA synthetase (acetate: CoA ligase, adenosine monophosphate [AMP], E. C. 6. 2. 1. 1) and acyl CoA synthetases (acid: CoA ligase, AMP, E. C. 6. 2. 1. 2 and 6. 2. 1. 3) were assayed by a method partially modified from that of Samuel and Ailhaud (14). The reaction mixture contained in a final volume of 0.4 ml, 60 μmol Trihydroxymethylaminomethane (Tris)-HCl buffer (pH 7.5), 5 μmol MgCl₂, 5 μmol adenosine triphosphate (ATP), 1.25 μmol Coenzyme A (CoA), 1 μmol dithiothreitol (DTT), 70 μmol hydroxylamine, 0.5 μmol 14C-labeled fatty acid (0.6 Ci/mmol) and 1.5 mg protein from the bacillary extract. After incubation at 37°C for 15 minutes, the reaction was stopped by acidifying with 1.0 ml 2% perchloric acid, then the excess fatty acid was eliminated by washing with diethyl ether (with six 5 ml portions of ether to eliminate free fatty acids in case of acyl CoA synthetase, with four 5 ml portions of ether to eliminate free fatty acids in case of acyl CoA synthetase). A half milliliter of the aqueous phase was pipetted into a counting vial, mixed with 10 ml Insta-Gel (a liquid scintillation cocktail for aqueous solutions, Packard Co. Ltd.), then counted with Packard’s liquid scintillation counter. Model 3385. Acetyl CoA carboxylase (acetyl CoA: carbon-dioxide ligase, adenosine diphosphate [ADP], E. C. 6. 4. 1. 2) was assayed by counting radio-activity in malonyl CoA formed from NaH14CO₃ and acetyl CoA according to the method of Ernle (14). Assay of de novo fatty acid synthetase was carried out by the counting of radioactivity in fatty acids produced from 2-14C-malonyl CoA and acetyl CoA according to the method of Vance et al (18), except for addition of 2,6-di-o-methyl...
β-cyclodextrin, one of the special stimulators for mycobacterial fatty acid synthetase (1), in place of mycobacterial polysaccharides (16), in the reaction mixture. Acetyl CoA dependent acyl CoA elongation was assayed by using a method partially modified from that of Hinsch and Seubert (3). The reaction mixture contained in a final volume of 1.0 ml, 100 μmol N-trihydroxymethyl-2-aminoethane sulfonic acid (TES)-KOH buffer (pH 5.6), 1 μmol DTT, 0.5 μmol reduced nicotinamide adenine dinucleotide (NADH), 0.06 μmol acyl CoA (C₄-C₁₆) as shown in Table 3, 0.15 μmol 1-14C-acetyl CoA (0.4 Ci/mol) and 1 mg protein from the bacillary crude extract. The reaction mixture was incubated at 37 °C for ten minutes, then 0.3 ml 50% KOH solution was added to it. The mixture was saponified for 30 minutes in a boiling water bath followed by acidification with 0.5 ml of 6 N HCl. The acidified mixture was extracted three times with n-pentane (acetic acid is not extracted with n-pentane under these conditions). Radioactivity in the n-pentane extract thus obtained was counted by the liquid scintillation counter.

Identification of fatty acids produced by acetyl CoA dependent acyl CoA elongation system. In order to identify fatty acids produced by the acetyl CoA dependent acyl CoA elongation system, a three times larger scale of reaction mixture than that of the ordinary assay was used. The n-pentane extract obtained in this case was evaporated to dryness, then the residual radioactive fatty acid mixture was methylated with 15% (W/V) BF₃ in methanolic solution (1). Identification of newly formed individual fatty acids by the elongation system was carried out by radio-gaschromatography, using Packard's proportional counter, Model 894 connected to Packard's gas liquid chromatographic apparatus. Model 8067.

Chemicals. Sodium 2-14C-acetate, sodium 2-14C-propionate, sodium 1-14C-butyrate, sodium 1-14C-octanoate, 1-14C-palmitic acid, sodium 14C-bicarbonate and 1-14C-acetyl CoA were purchased from the Radiocchemical Center, Amersham. 2-14C-malonyl CoA was purchased from New England Nuclear, Boston. Acetyl CoA, propionyl CoA, butyryl CoA, octanoyl CoA, decanoyl CoA, lauryl CoA, palmitoyl CoA and stearoyl CoA were purchased from P. L. Biochemicals, Milwaukee. 2,6-di-O-methyl β-cyclodextrin was a generous gift from Dr. A. Kawaguchi, Institute of Applied Microbiology, University of Tokyo. All other chemicals were reagent grade from commercial sources.

RESULTS

Acetyl CoA synthetase and acyl CoA synthetase activities. As shown in Table 1, it was found that the specific activity of acetyl CoA synthetase in the extract of M. lepraemurium was found to be about fivefold lower than that of M. smegmatis. Acyl CoA synthetase activities of M. lepraemurium for fatty acids with various chain lengths (C₃-C₁₆) were compared also with those of M. smegmatis, as shown in Table 1.

Acetyl CoA carboxylase activities. As shown in Table 2, activity of acetyl CoA carboxylase and de novo fatty acid synthetase in crude extracts of M. lepraemurium and M. smegmatis.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity of acetyl CoA synthetase and acyl CoA synthetase (nmol/mg protein/min at 37°C)</th>
<th>M. lepraemurium</th>
<th>M. smegmatis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-14C-Acetate</td>
<td>1.87</td>
<td>10.93</td>
<td></td>
</tr>
<tr>
<td>2-14C-Propionate</td>
<td>0.20</td>
<td>3.47</td>
<td></td>
</tr>
<tr>
<td>1-14C-Butyrate</td>
<td>0.33</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>1-14C-Octanoate</td>
<td>0.13</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>1-14C-Palmitate</td>
<td>3.47</td>
<td>14.93</td>
<td></td>
</tr>
</tbody>
</table>

| Specific activity of acetyl CoA carboxylase and de novo fatty acid synthetase in crude extracts of M. lepraemurium and M. smegmatis. |
|---------------------------------------------------------------------------------------------------------------|-----------------|--------------|
| Acetyl CoA carboxylase                                                                                       | undeletable     | 7.20         |
| De novo fatty acid synthetase (nmol malonyl CoA/mg prot/min at 37°C)                                       | trace           | 1.16         |
| (less than malonyl CoA/mg prot/min at 37°C)                                                                | 0.10            |              |
TABLE 3. Specific activity of acetyl CoA dependent acyl CoA elongation system in crude extracts of M. lepraemurium and M. smegmatis.

<table>
<thead>
<tr>
<th>Starting acyl CoA</th>
<th>M. lepraemurium</th>
<th>M. smegmatis</th>
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<tr>
<td>Butyryl CoA</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td>Octanoyl CoA</td>
<td>0.90</td>
<td>0.67</td>
</tr>
<tr>
<td>Decanoyl CoA</td>
<td>0.76</td>
<td>0.76</td>
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<tr>
<td>Lauril CoA</td>
<td>0.72</td>
<td>0.27</td>
</tr>
<tr>
<td>Palmitoyl CoA</td>
<td>0.42</td>
<td>0.13</td>
</tr>
<tr>
<td>Stearyl CoA</td>
<td>0.29</td>
<td>0.09</td>
</tr>
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</table>

Specific activity (nmol acetyl CoA/mg prot. min at 37°C).

Boxylase in the extract of M. lepraemurium could not be detected, under the conditions used, whereas in the activity of this enzyme in the extract of M. smegmatis it was found that 7.20 nmol NaH14CO3 reacted to form malonyl CoA per mg protein per minute at 30°C.

De novo fatty acid synthetase activities.

As shown in Table 2, activity of de novo fatty acid synthetase in the extract of M. lepraemurium was too weak to measure precisely whereas in the activity of this enzyme in the extract of M. smegmatis was found to be 1.16 nmol malonyl CoA incorporated into fatty acids per mg protein per minute at 37°C.

Acetyl CoA dependent acyl CoA elongation. There has been good evidence that Mycobacterium tuberculosis (H37Rv) retains an avidin-insensitive acetyl CoA dependent acyl CoA elongating system (17). For ascertaining whether a similar system is also capable of existence in M. lepraemurium as well as M. smegmatis, enzyme activities participating in acetyl CoA dependent acyl CoA elongation in these two mycobacterial extracts were investigated. In consequence, this system could be detected in both mycobacterial extracts, and specificity of these elongation systems for various acyl CoA (C4-C18) were found to be similar to each other as shown in Table 3. These elongation systems were clearly distinguishable from de novo fatty acid synthesis because the following features could clearly discriminate the former from the latter: 1) an avidin-insensitivity (even 100 μg/ml avidin in the incubation medium was almost insensitive) for the elongation systems; 2) the specificity of elongation for various chain length's acyl CoA (as shown in Table 3, the most available acyl CoA to the elongation system was octanoyl CoA or decanoyl CoA); 3) no requirement of reduced nicotinamide adenine dinucleotide phosphate (NADPH) but an absolute requirement of NADH for the elongation systems; 4) optimum pH 5.6 pH for the elongation systems [it is 7.0 for the de novo fatty acid synthetase of M. smegmatis (18)]. The products formed in the reaction mixture for the acetyl CoA dependent acyl CoA elongation system in the extract of M. lepraemurium were identified by radiogaschromatography with authentic samples. As shown in Figure 1, radioactive lauric and myristic acids could be identified.
in the reaction mixture, including 1-14C-acetyl CoA and decanoyl CoA as substrates. Two other radioactive peaks near that of lauric acid in Figure 1 were presumed to be some intermediates occurring during the elongation of decanoyl CoA but not yet identified definitely. In addition, as shown in Figure 2, radioactive stearic acid as well as lignoceric acid could be detected in the reaction mixture for the elongation system in the extract of M. lepraemurium, containing 1-14C-acetyl CoA and palmitoyl CoA as substrates. Other highly radioactive peaks near that of stearic acid in Figure 2 are not yet identified. Products from the acetyl CoA dependent acyl CoA elongation system in the extract of M. smegmatis were also identified by radiogaschromatography and it was found that the main products were similar to those in the case of M. lepraemurium. In attempts to elucidate a physiologic role for the acetyl CoA dependent acyl CoA elongation system in M. lepraemurium, its intact cells were incubated with 1-14C-palmitic acid, from which elongated fatty acids during the incubation were followed by radiogaschromatography.
In consequence, a newly formed radioactive stearic acid was clearly detected as shown in Figure 3. Other radioactive peaks near that of stearic acid in Figure 3 are not yet identified.

**DISCUSSION**

In comparing the specific activity of enzymes pertaining to the biosynthesis of fatty acids in crude extracts of *M. lepraemurium* (an extremely slow grower) and of *M. smegmatis* (a rapid grower), it was found that these enzymes could be classified into three groups with respect to differences in their activity between these two mycobacteria. That is: 1) enzymes whose activities differ conspicuously from each other (acyetyl CoA carboxylase and de novo fatty acid synthetase); 2) enzymes whose activities are close enough (enzymes pertaining to the acetyl CoA dependent acyl CoA elongation system); and 3) enzymes whose activities differ moderately (acyetyl CoA synthetase and acyl CoA synthetase).

Acetyl CoA carboxylase and de novo fatty acid synthetase, belonging to the first group described, are both fundamental enzymes for biosynthesis of fatty acids among diverse living organisms. The activity of de novo fatty acid synthetase in a crude extract of *M. smegmatis* was reported by Bloch's group (11), that is 1.1 nmol malonyl CoA incorporated in fatty acids per mg protein per minute at 37°C which is almost identical with that in our case (Table 2). On the other hand, the activity of acetyl CoA carboxylase in a crude extract of *M. smegmatis* was also reported by Erfle (6), that is 26.3 nmol K HCO_3 incorporated in malonyl CoA per mg protein per minute at 30°C which is about 3.5-fold higher than that in our case (Table 2). Though the reason for this discrepancy is unclear, it seems highly improbable that the activity of acetyl CoA carboxylase as well as de novo fatty acid synthetase in the extract of *M. lepraemurium* were missed or too little evaluated due to technical errors on our part. The reason why activities of these two enzymes in the extract of *M. lepraemurium* are so tenuous is unclear at present. Recently, it was reported that the cellular content of acetyl CoA carboxylase in *Saccharomyces cerevisiae* is reduced by the addition of long chain fatty acids to the culture medium (8). In addition, it has also been reported that endogenous fatty acid biosynthesis in two yeast species, *S. cerevisiae* and *Candida lipolytica* is completely repressed by the addition of long chain fatty acids to the growth medium (11). Though the possibility of similar phenomena could be inferred in the case of the cultivation of *M. lepraemurium* on Ogawa's yolk medium, it is very hard to evaluate this possibility because growth media other than Ogawa's are unknown up to the present.

Acetyl CoA dependent acyl CoA elongation system could be detected in *M. lepraemurium* and *M. smegmatis* for the first time. The general enzymatic properties of this system in these two mycobacteria appeared similar to each other. Further study on enzymatic properties of this system in *M. smegmatis* are being carried out in our laboratory. There seems to be good evidence that this system may be fairly common among mycobacteria, because a similar system has also been found in *M. tuberculosis* H_37Ra (12).

From the relatively high activity of acetyl CoA dependent acyl CoA elongation system in the extract of *M. lepraemurium* as shown in Table 3 as well as the evidence concerning elongation of palmitic acid occurring in vivo as shown in Figure 3, it seems reasonable to assume that the acetyl CoA dependent acyl CoA elongation system has a physiologic function in synthesizing long chain fatty acids in *M. lepraemurium*. On the other hand, it may be imagined that de novo synthesis of fatty acids has little significance for supplying fatty acids to *M. lepraemurium*. From the results shown in Table 2. Such low activity of de novo synthesis of fatty acids in *M. lepraemurium* has never been reported among any other cultivable mycobacteria. These findings suggest that the extremely low activity of de novo synthesis of fatty acids in *M. lepraemurium* may cause a severely limited supply of fatty acids which are necessary in profusion at the moment of cell multiplication, to this microorganism. It is obvious that other factors such as the peculiar electron transfer system of *M. lepraemurium* as reported by Mori (9, 10) may also account for the extremely sluggish growth of this mycobacterium.

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*Mycobacterium phlei*, KCTC 356 used in these studies has recently been corrected to be a strain of *M. smegmatis* by the original authors (11).
SUMMARY

In comparing the specific activity of enzymes pertaining to the biosynthesis of fatty acids in crude extracts of cultivated M. lepraemurium and M. smegmatis, it was found that:

1. The activity of acetyl CoA carboxylase of the former organism was undetectable and that of de novo fatty acid synthetase was too weak to measure exactly, under the condition used, whereas both activities of the latter organism were comparable to those already reported by other authors.

2. The activity of acetyl CoA dependent acyl CoA elongation system of M. lepraemurium was relatively high and close to that of M. smegmatis.

3. The activities of acetyl CoA synthetase and acyl CoA synthetase of M. lepraemurium were moderately lower than those of M. smegmatis.

The relation between this peculiar fatty acid synthesizing enzyme system of M. lepraemurium and its extremely sluggish growth is discussed.

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