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A FATTY ACID-BINDING PROTEIN OF *MYCOBACTERIUM SMEGMATIS*

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Abstract

A protein, binding especially fatty acids of 16∼18 carbons, was found in a soluble fraction of *Mycobacterium smegmatis*, and it was purified about 690-fold by a heat-treatment, ammonium sulfate precipitations, gel filtrations through Sephadex G-50 and DEAE-cellulose column chromatography. Sodium dodecyl sulfate polyacrylamide electrophoresis revealed that this protein exists in the form of a single polypeptide chain with a molecular weight of 7,800. The order of binding ability of this protein to fatty acids were oleic acid, palmitoyl-CoA and palmitic acid in terms of quantity of fatty acids found per mg protein.

INTRODUCTION

Two kinds of proteins binding various organic ions were found in a soluble fraction of rat liver by Levi et al. in 1969[1]. Thereafter, one of these proteins, nominated as Z protein, was purified to homogeneity[2], and shown to bind specifically long-chain fatty acids[3,4], and their CoA thioesters[5]. The Z protein may be similar to another protein (FABP), which binds long-chain fatty acids, isolated from the soluble fraction of rat intestinal mucosa[6]. A binding activity of myoglobin of rat to a long-chain unsaturated fatty acid[7] and butyrate binding protein (BBP) in the liver of rat and mouse, binding specifically short-chain fatty acids[8], were also reported.

On the other hand, any fatty acid-binding protein from bacterial origin has not yet been reported at present. Among many kinds of micro-organisms, mycobacteria are well-known to be preeminently rich in several kinds of lipids, including triglyceride and mycolic acid, etc., and various fatty acid synthesizing enzyme systems, including multienzyme complex of fatty acid synthetase[9,10], malonyl-CoA dependent fatty acid elongation system[11,12] and acetyl-CoA dependent fatty acid elongation system[13,14], are also found in mycobacterium. Accordingly, a possibility of existance of some fatty acid-binding proteins in mycobacteria was imagined and investigated using *Mycobacterium smegmatis*,

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and some confirmative results as to such protein were obtained and presented in this paper.

MATERIALS AND METHODS

1) *Preparations of the bacterial soluble fraction.* The strain of *M. smegmatis* used and the cultivation method were the same as reported previously[15]. The bacilli withdrawn at the stationary phase (5～6 days after inoculation) were washed thoroughly with water, suspended in 10 mM potassium phosphate buffer (pH 7.4) "the phosphate buffer" and then broken up in a French pressure cell (Ohtake Co.) at 500 kg/cm^2. After centrifugation at 20,000 x g for 30 min, the supernatant was centrifuged at 230,000 x g for 3 hrs. Streptomycin (1 mg/1 mg protein) was added to the supernatant, and the precipitate formed by nucleic acid was discarded.

2) *Assay for fatty acid-binding activity.* The fatty acid-binding activity was measured by nonequilibrium gel filtration of the protein on Sephadex G-25 columns. Incubation mixture (1 ml) containing 2 0 μmol the phosphate buffer, 25 μmol KCl, 10 mnol [1-^{14}C] substrate and 3 ～ 15 μg protein was incubated at 0°C for 20 min, and then applied to a Sephadex G-25 column (fine, 1.1 × 19 cm). The elution was carrried out at 4°C at a flow rate of 24 ml/h with a solution containing 0.015% triton WR 1339, 25 mM KCl and 20 mM the phosphate buffer. Radioactivity in the fraction eluted at void volume was measured with Packard' s liquid Scintillation Counter (type 2450) using a toluene base scintillator containing 33% Triton X-100 (v/v). For calculation of nanomoles of [1-^{14}C] substrate bound to the protein, corrections were made by subtracting the value from a control experiment without the protein.

3) *Other procedures.* Polyacrylamide disc gel electrophoresis was carried out by the method of Davis[16]. The protein sample was loaded on a top of gel containing 7.5% acrylamide and a constant current of 3 mA per tube was supplied. The molecular weight of subunit was determined using Combithek® (Cat. No. 236292, Boehringer Mannheim bH•Biochemica) which contains calibration proteins in range of 3,000 to 22,000[17,18]. For a SDS-disc electrophoresis, the sample protein was mixed with 10 mM sodium phosphate buffer (pH 7.2) containing 0.2% SDS, 10% glycerol (v/v) and 3% mercaptoethanol (v/v), heated at 90°C for 3 min, then loaded on the top of gel containing 10% polyacrylamide, 0.1% SDS and 0.1M sodium phosphate buffer (pH 7.2). A constant current of 8 mA per tube was supplied for about 3 hrs. The gels were removed and stained with 0.5% Coomassie brilliant blue. Protein was determined by the method of Lowry et al. using bovine serum albumin as a standard[19].

4) *Chemicals.* [1-^{14}C] fatty acids and [1-^{14}C] palmitoyl-CoA were purchased from the Radio-chemical Centre, Amersham. Sephadex G-25 and G-50 were from Pharmacia Fine Chemicals. All other Chemicals were reagent grade.

RESULTS

1) *Detection of fatty acid-binding protein in the soluble fraction.* Fifty μl of [1-^{14}C] palmitic acid (3.0×10^6 cpm, 37.5 nmol) dissolved in 0.3% Triton WR 1339 was mixed at 0°C with 1.95 ml of mixture containing 20 mg protein of the soluble fraction as described above, 0.3 mmol KCl and 20 μmol the phosphate buffer. Ten minutes later all the mixture was then applied to a column of Sephadex G-50 (fine, 2.1×46 cm) and eluted with "the phosphate buffer" containing 0.15 M KCl at flow rate of 34 ml/h. Five-ml was collected for each fraction, and its protein content and radioactivity were measured. The results obtained are shown in Fig. 1. As shown, a conspicuous radioactive peak was eluted around the 33th fraction. About 23%

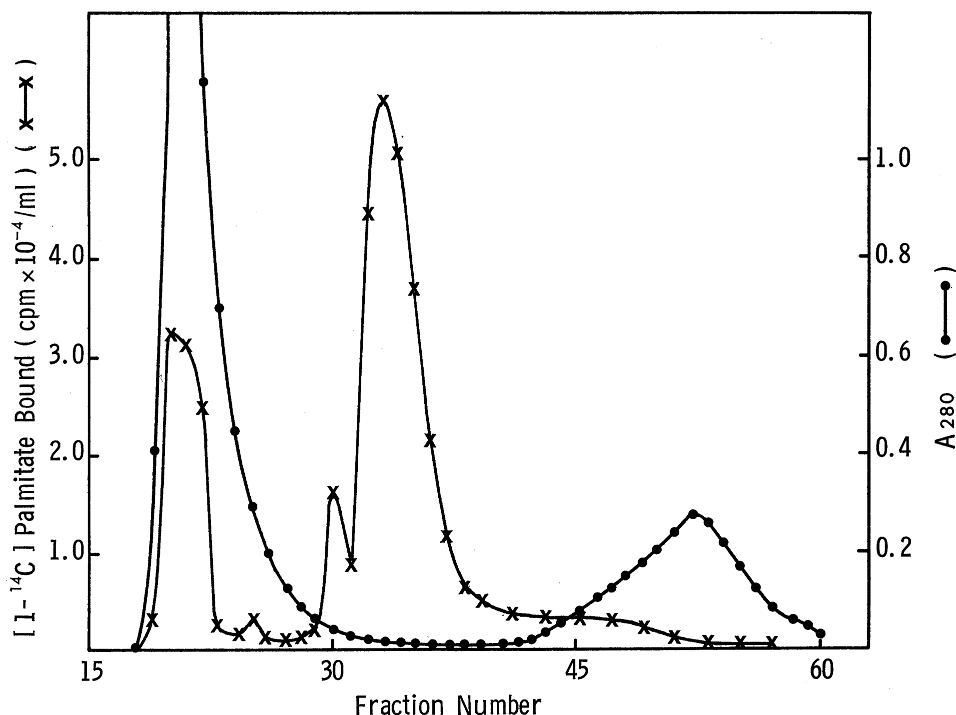

Fig. 1. Gel filtration of *M. smegmatis* 230,000×g supernatant with [1-^{14}C] palmitate on Sephadex G-50. All operations were carried out as described in the text.

of the total radioactivity applied to the column was recovered in this peak, though about 9.4% was also found in a void volume.

2) *Purification of the fatty acid-binding protein.* The soluble fraction of *M. smegmatis* prepared as described above was heated at 50°C for 10 min and the denaturated proteins were removed by centrifugation. To the supernatant thus obtained was added ammonium sulfate to make 60% saturation. The formed precipitate was discarded by centrifugation, and the supernatant was saturated 100% with ammonium sulfate. The precipitate formed after standing at 4°C for 12 hrs was collected and dissolved in "the phosphate buffer" and then applied to a column of Sephadex G-50 (fine, 3.1×46 cm, void volume, 109 ml). The elution was carried out with "the phosphate buffer" as used for dissolving the sample. The fractions of elution volume, 168∼204 ml, being expected to hold the palmitic acid-binding protein from the experiment of Fig. 1, were collected, and concentrated by lyophilization. This sample was deionized using Sephadex G-25, and then applied to a column (1.6×8 cm) of DEAE-cellulose equibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was eluted with 10 mM Tris-HCl buffer (pH 8.0) containing a linear gradient concentration of NaCl from 0 to 0.12 M (total volume, 101 ml). Each 2.5 ml of the eluate was collected at a flow rate of 24 ml/h and the palmitate binding activity and optical density at 280 nm were measured. The results were shown

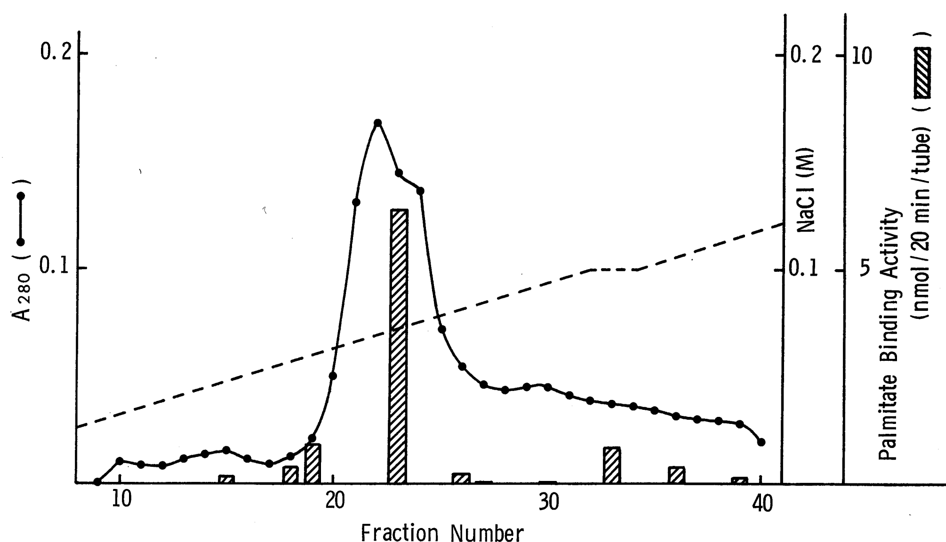

Fig. 2. DEAE-cellulose column chromatography of the pooled fractions from the Sephadex G-50 step. All operations were carried out as described in the text.

TABLE 1.
Purification of the FABP of *Mycobacterium smegmatis*

Purification step	Total protein (mg)	Specific activity (nmol/mg/20min)	Yield (%)	Purification (-fold)
230,000 x g Supernatant	16,400	0.81	100	1.00
Streptomycin	16,300	0.70	85.9	0.87
50°, 10 min	10,600	0.86	68.6	1.06
60-100% $(NH_4)_2SO_4$	1,610	2.47	29.9	3.05
Sephadex G-50 (I)	28.7	114	24.4	140
DEAE-cellulose	3.60	226	6.1	280
Sephadex G-50 (II)	1.46	525	5.8	650
55°, 10 min	1.36	555	5.7	687

in Fig. 2. Fractions (No. 18∼25) containing mainly palmitate binding protein were pooled, and concentrated as before, then applied to a column of Sephadex G-50 (fine, 2.1×46 cm, void volume, 57.7 ml). The column was eluted by " the phosphate buffer " and the fractions (elution volume, 91.3∼107.5 ml), which was expected to hold the main part of binding protein as described

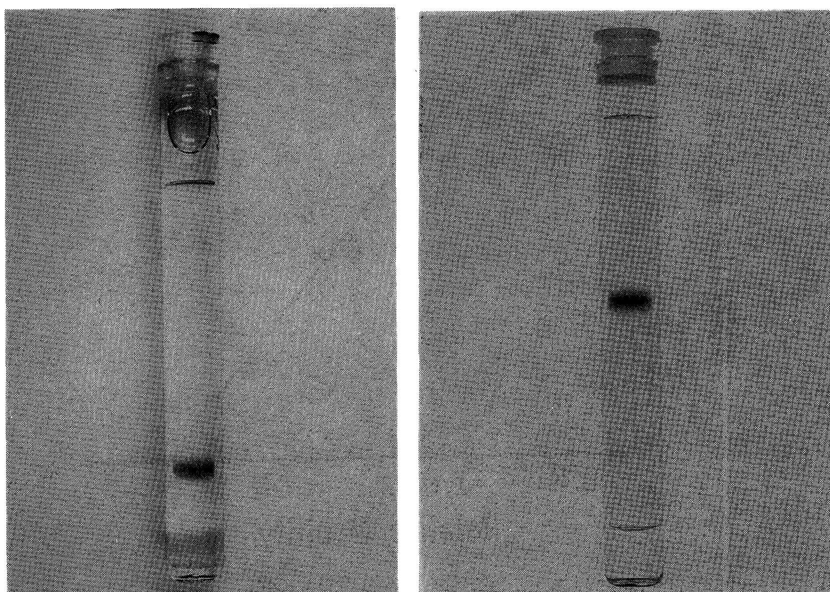

Fig. 3. Native and SDS-polyacrylamide disc gel electrophoresis of the FABP. Right native polyacrylamide gel of 17 μg ; left SDS-polyacrylamide gel of 10 μg of the FABP. Electrophoresis and gel staining were carried out as described in MATERIALS & METHODS.

above, were collected. These fractions were concentrated, deionized by gel filtration as above, and then stored below -20°C. The sample protein was homogeneous at this step as shown below but sometimes, it was necessary to heat the sample at 55°C for 10 min to eliminate the unpure parts. The course of purification is shown in Table 1. As shown, the fatty acid-binding protein (the FABP) was purified about 140-fold at the step of Sephadex G-50 (I), finally about 690-fold but the final yield of binding activity was about 5.7%.

3) *Molecular size of the FABP.* As shown in Fig. 3, when 17 μg of the FABP was subjected to 7.5% polyacrylamide disc gel electrophoresis, a single protein band was observed after staining with Amido black (right). When 10 μg of the FABP was subjected to 0.1% SDS-7.5% polyacrylamide disc gel electrophoresis, a single protein band was also observed after staining Coomassie brilliant blue (left). This result suggests that the FABP is composed of monomer or only a single subunit. Determination of the subunit molecular weight of the FABP gave approximately 7,800 as shown in Fig. 4. It was

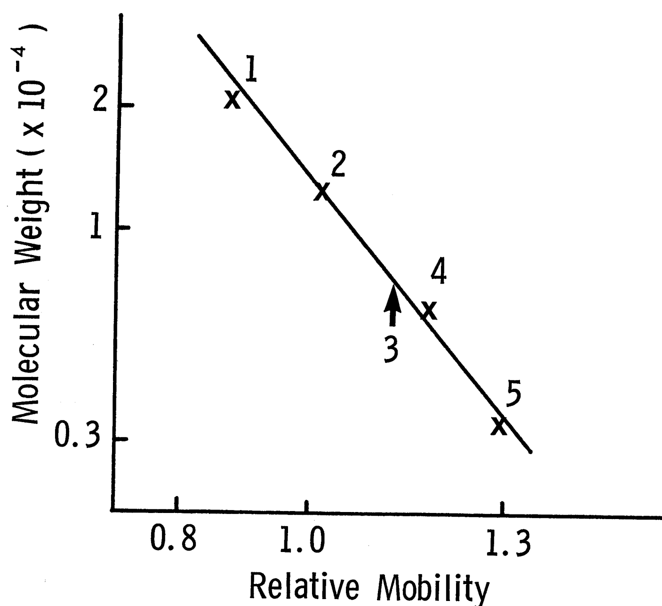

Fig. 4. Determination of the subunit molecular weight of the FABP by SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out as described in MATERIALS & METHODS. Four calibration proteins with the following molecular weights were run with the FABP. 1, soybean trypsin inhibitor (21,500); 2, cytochrome c (12,500); 3, FABP; 4, aprotinin (6,500); 5, insulin chain B (3,400).

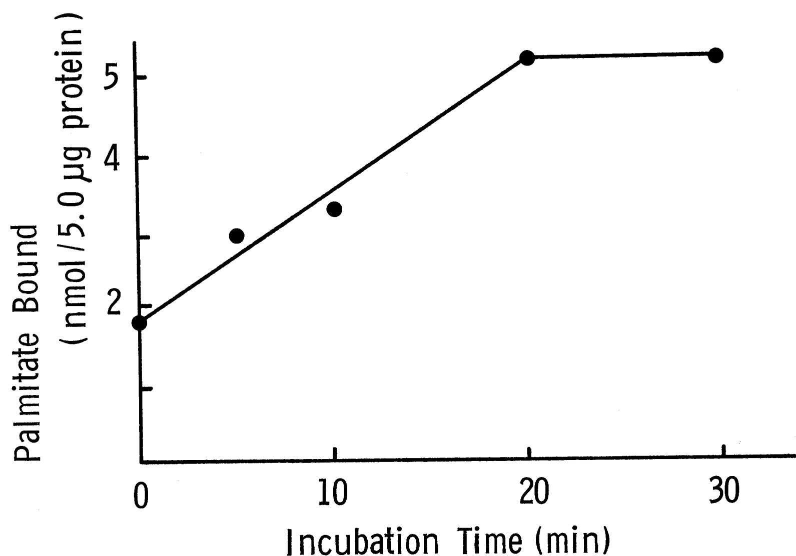

Fig. 5. Time course of palmitate binding activity of the FABP. The binding assay was carried out as described in MATERIALS & METHODS except that incubation time was varied.

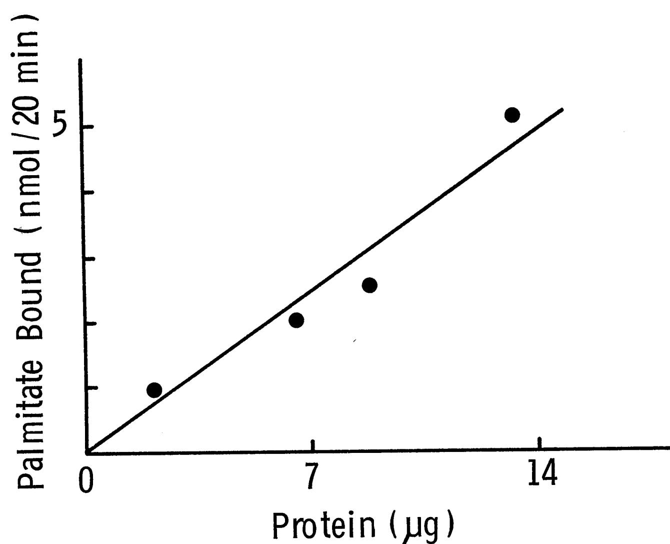

Fig. 6. Palmitate binding activity of the FABP as a function of protein content. The binding assay was carried out as described in MATERIALS & METHODS except that the FABP content was varied.

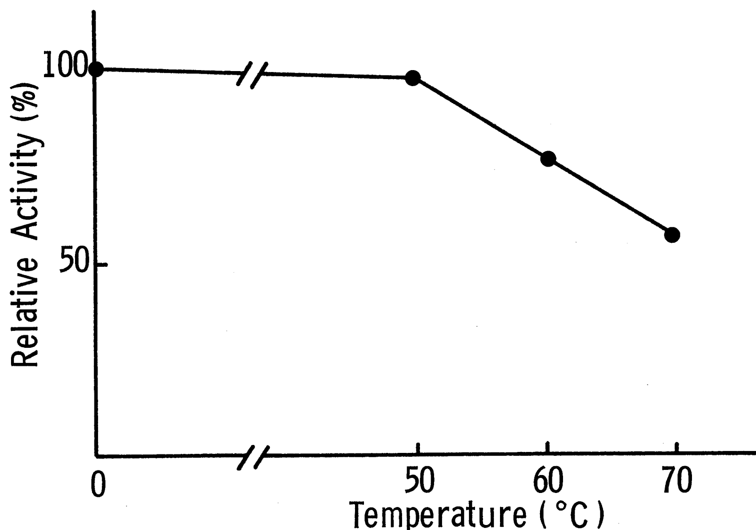

Fig. 7. Effect of heating for 10 min on palmitate binding activity. The binding assay was carried out as described in MATERIALS & METHODS.

also found that native molecular weight of the FABP was smaller than that of cytochrome *c* (12,500) by gel filtration on Sephadex G-50. These results are indicating that the FABP is a monomer having the molecular weight of 7,800.

4) *Some Properties of the FABP for palmitate binding activity.* The effect of incubation time on the binding activity is shown in Fig. 5. The binding occurred not only instantly but also gradually with time up to 20 min at 0°C. The incubation temperature (0~20°C) was not so effective on the binding activity (data not shown). The pH optimum for the binding activity was 7.0 to 7. 8. Fig. 6 shows a proportionality of the binding activity to the concentration of the FABP up to 15 μg of protein. Fig. 7 shows effects of heating temperature (for 10 min) before the incubation on the binding activity. The binding activity seemed stable enough against heating till 50°C, but about 48% decrease was found by heating at 70°C.

5) *Binding specificity of the FABP.* Table 2 shows the binding specificity of the FABP. Butyrate and octanoate scarcely bound to the FABP. Myristate bound to the FABP by about 45% of palmitate under the same condition. Oleic acid and palmitoyl-CoA bound to the FABP about 4.5 and 4.1 times more than palmitic acid respectively.

TABLE 2.
Binding of fatty acids and fatty acyl-CoA to the FABP

Substrates	Binding activity	
	nmol/8.8μg protein/20min	%
Butyrate	0	0
Octanoate	0	0
Myristate	0.42	44.5
Palmitate	0.93	100
Oleate	4.24	454
Palmitoyl-CoA	3.85	412

The binding assay was carried out as described in MATERIALS & METHODS.

DISCUSSION

Some fatty acid-binding proteins such as BBP[8], FABP[6] and Z protein[5] have been already purified from animal tissues. BBP, composed of subunits of 45,000–48,000 dalton, was shown to bind especially butyric acid and other short-chain fatty acids, and this binding activity was completely lost at 80°C for 1.5 min[8]. The molecular weight of Z protein and FABP purified from rat intestinal mucosa are both estimated to be 12,000. It was also found that Z protein is heat-stable[1] and binds long-chain fatty acids such as oleic acid and palmitic acid[2,3], and their CoA thioesters[4]. FABP was also found to bind long-chain fatty acids[6]. Another protein binding especially oleic acid was also found recently in heart and red skeletal muscle of rat and mouse. Its molecular weight was estimated to be 16,000, and it was thought to be identical to myoglobin[7]. Among these fatty acid-binding proteins from animal sources, the Z protein and the intestinal FABP appeared most similar to the FABP of *M. smegmatis* from the viewpoints of molecular size, binding specificity, heat-stability, etc.

It has been clarified that Z protein and FABP purified from intestinal mucosa are involved in the lipid metabolism[5,20,21,22,23] and the intracellular fatty acid transport[24,25]. Physiological role of the FABP isolated from *M. smegmatis* is left to be investigated in future.

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