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Expression in *Escherichia coli* and characterization of the fatty-acid-binding protein from human muscle

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The coding part of the cDNA encoding human muscle fatty-acid-binding protein (FABP) was ligated in the pET8c vector and expressed under the control of the lacUV5 promoter. After induction with isopropyl β-D-thiogalactopyranoside, almost 12% of the cytoplasmic proteins consisted of FABP. The protein could be isolated after sonication of the bacterial pellet followed by $(NH_4)_2SO_4$ precipitations, anion-exchange chromatography and gel filtration. The muscle FABP produced in *Escherichia coli* has an isoelectric point of 5.3 and is recognized by anti-(human muscle FABP) antiserum after Western blotting. The purified FABP has a preference for binding to palmitic acid and C_{18}–C_{22} (poly)unsaturated fatty acids, and no affinity to palmitoyl-CoA or other hydrophobic ligands tested. The dissociation constant for oleic acid is 0.58 μM, with a binding stoichiometry of 0.72 mol of fatty acid/mol of protein. The physicochemical and binding characteristics of the protein were in complete agreement with those of FABP isolated from human skeletal muscle.

INTRODUCTION

In mammalian cells a class of small closely related proteins, the so-called fatty-acid-binding proteins (FABPs), are thought to be involved in fatty acid metabolism [1–3]. Although many properties of these proteins have been demonstrated *in vitro*, their precise role in cell metabolism remains to be established. It is thought that the FABPs can act either as transfer proteins of fatty acids in cytoplasm or as an internal pool of fatty acids [1–3].

On the basis of cDNA and/or protein structures, we know that there are at least five different types of FABP in man, which have 25–65% sequence identity [3]. Based on their initial site of isolation they have been named liver, heart, intestinal, adipocyte and myelin FABP [2,3]. The existence of different FABP types suggests a specific adaptation of these proteins, which probably originate from a common ancestor gene. In some tissues, e.g. intestine [1,4], kidney [5,6] and stomach [7], several types of FABP have been found. The localization of a specific FABP type in these tissues is related to tissue region, cell type and development. It was suggested that one type has a more specific function related to fatty acid metabolism, whereas the other types have a more general function and bind various kinds of hydrophobic ligands [3]. In human muscle tissue, two types of FABP were thought to be present, one type in heart [8] and the other type in skeletal muscle [9]. The two FABPs could be slightly different from each other, like the bovine heart and brain FABPs [10,11], because of a functional and/or tissue-specific adaptation. Recently, however, the amino acid sequence of human skeletal muscle FABP was elucidated by cDNA sequencing and peptide analysis [12], and appeared to be completely identical to that of human heart FABP [8].

Until now, cDNAs encoding the FABP types from rat intestine [13,14] and liver [15], bovine heart [16,17], mouse adipocyte [18] and pig ileal epithelium [19] have been expressed in either *Escherichia coli* or yeast. In this way, large amounts of specific FABPs could be obtained. They were then used for crystallization studies to elucidate the tertiary structure by Röntgen diffraction [14,15,19–21] or for ^{13}C-n.m.r. studies [19,22] of the ligand–protein interaction.

In this report we describe the construction and expression of a new vector containing the cDNA encoding human muscle FABP in *E. coli* and the purification of the protein from the bacterial lysate. The physicochemical and binding properties, which identify the protein as a true human muscle FABP, are also demonstrated.

MATERIALS AND METHODS

Isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from Research Organics Inc., Cleveland, OH, U.S.A.; DEAE-32 cellulose was from Whatman Ltd., Maidstone, Kent, U.K.; Sephadex G-50 was from Pharmacia Biotechnologies, Uppsala, Sweden; and [1-^{14}C]oleic acid was from Amersham, Little Chalfont, Bucks., U.K. The origin of ligands was described in [23]. Human muscle FABP and its antiserum were prepared and characterized as described [9]. All other chemicals were of analytical grade.

Cloning of the coding sequence of muscle FABP into pET8c

Two different oligonucleotides were used in a PCR experiment to create appropriate ligation sites on the cDNA insert encoding human muscle FABP [12] for ligating it into the pET8c vector [24]. At the start codon a unique *Nco*I site was created with the oligonucleotide 5′-GCCAGCATCA*CCATGG*TGGACGCTT-TC-3′, and just after the stop codon a unique *Bam*HI site was created with 5′-ATCACCAGT*GGATTC*AGGTCATGCCTC-3′. After purification of the *Nco*I/*Bam*HI mutant, this novel cDNA was digested with both restriction enzymes and ligated in pET8c [25].

Expression of FABP in *E. coli*

Bacteria BL21 (DE3) were transformed with pET8c containing the mutant cDNA under standard conditions [26]. A 5 ml overnight culture was used to inoculate 3 litres of 2 × TY medium (1.6% bactotryptone/1% yeast extract/0.5% NaCl) and 240 mg of ampicillin/l. Bacteria were grown under shaking at 37 °C. At an A_{550} of 0.5 the culture was induced with 0.5 mM-IPTG and

Abbreviations used: FABP(s), fatty-acid-binding protein(s); IEP, isoelectric point; IPTG, isopropyl β-D-thiogalactopyranoside.
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incubated for 3 h at 37 °C. Bacteria were harvested by 10 min of centrifugation at 5000 *g* and the pellet was washed with 100 ml of 150 mM-NaCl/5.4 mM-sodium phosphate/1.3 mM-potassium phosphate (pH 7.4). The pellet was resuspended in 50 ml of 50 mM-Tris/HCl (pH 8.0)/10 % sucrose and sonified for 3 × 30 s with the broad tip of a Branson Sonifier. The suspension was centrifuged at 30000 *g* for 30 min, $(NH_4)_2SO_4$ was added to the supernatant to 50 % saturation and the mixture was stirred for 1 h at 0 °C. The suspension was centrifuged for 25 min at 30000 *g*, $(NH_4)_2SO_4$ was added to the supernatant up to 70 % saturation and the mixture was stirred for 1 h at 0 °C. After centrifuging the suspension for 25 min at 30000 *g*, the supernatant was dialysed overnight against 50 mM-Tris/HCl (pH 8.0). The dialysate was passed through a DEAE-cellulose column (5.5 cm × 4 cm) equilibrated in 50 mM-Tris/HCl (pH 8.0) and concentrated over an Amicon YM2 ultrafiltration membrane to 15 ml. Finally, 2.5 ml portions were loaded on a Sephadex G-50 column (110 cm × 2.5 cm) equilibrated in 5 mM-Tris/HCl (pH 8.0) and eluted with the same buffer.

Other procedures

Standard procedures were used for the gel electrophoresis, isoelectric focusing, immunoblotting, amino acid analysis and studies of [1-^{14}C]oleic acid binding, as previously described [9,23]. Competition studies of oleic acid binding were performed in 500 μl of 10 mM-Tris/HCl (pH 8.0) containing 0.67 μM-FABP, 1 μM-[1-^{14}C]oleic acid and 1 μM-ligand.

RESULTS

Expression of the human muscle FABP in *E. coli*

In order to obtain large amounts of human muscle FABP, we inserted the coding part of the human muscle cDNA into the pET8c expression vector (Fig. 1). The host strain of *E. coli* BL21(DE3) carries in its own DNA a bacteriophage (DE3) DNA fragment containing the *lacI* gene, the lacUV5 promoter and the T7 RNA polymerase gene [24]. Transcription of the T7 RNA polymerase gene is directed by the lacUV5 promoter, which can be induced by IPTG. The T7 RNA polymerase will transcribe the target DNA of the plasmid from the T7 RNA polymerase promoter ϕ10 (Fig. 1). To obtain an effective termination of transcription, a T7 DNA fragment containing the transcription termination signal Tϕ was ligated in the *Bam*HI site of the pET8c vector (Fig. 1). To enhance translation, the translation initiation signal s10 of the gene 10 protein (the major capsid protein of T7 polymerase) was ligated just downstream of the transcription initiation signal ϕ10 (Fig. 1). Initiation of translation starts immediately at the ATG of the *Nco*I cleavage site and therefore no fusion proteins will be obtained.

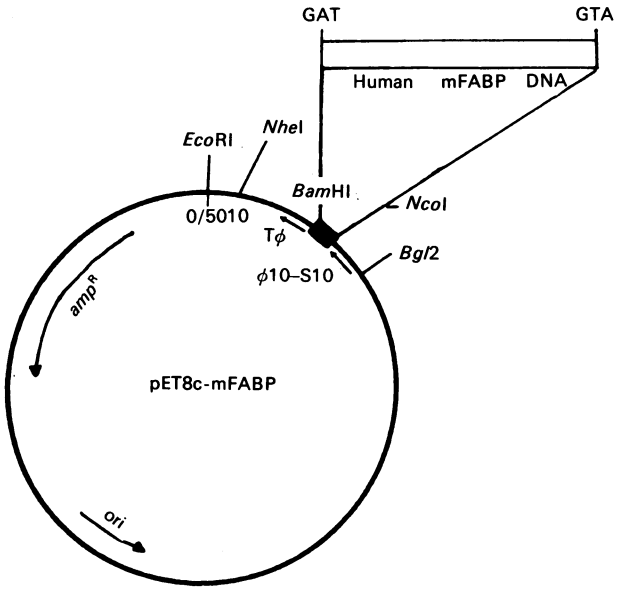

Fig. 1. Schematic drawing of the 5010 bp pET8c-muscle FABP expression vector used for synthesis of human muscle FABP in *E. coli*

The coding part of the human muscle FABP cDNA has been placed downstream of the transcription initiation signal ϕ10 between the translation initiation signal s10 and the transcription termination signal Tϕ. Transcription is regulated by the lacUV5 promoter which can be induced by IPTG. ori, origin.

Isolation and purification of muscle FABP from *E. coli*

Muscle FABP is effectively expressed in *E. coli* after induction with IPTG and has no obvious toxic effects on the bacterial cells. After 3 h of induction, 12 % of the cytoplasmic protein comprises muscle FABP (Fig. 2, lane 2). Because FABP is a water-soluble protein, we used a stepwise $(NH_4)_2SO_4$ precipitation to remove most of the prokaryotic proteins. After anion-exchange chromatography only a small amount of higher-molecular-mass proteins (30–70 kDa) contaminated the muscle FABP preparation (Fig. 2, lane 3). Finally, the protein was purified by gel filtration on Sephadex G50 (Fig. 2, lane 4). The yield of muscle FABP derived from *E. coli* was about 17 mg/litre of culture.

The protein has a molecular mass of about 15 kDa. Its pI estimated by isoelectric focusing was 5.3; the native protein from muscle had a pI of 5.2. The amino acid analysis of three different protein preparations was in good agreement with the amino acid composition obtained from muscle FABP isolated from human skeletal muscle [9] and the amino acid composition from cDNA data [12]. The procedure of Lowry *et al.* [27] gave a value of protein mass which was 1.5-fold higher than that determined by quantitative amino acid analysis.

On a Western blot it was shown that the total bacterial lysate did not contain muscle FABP before induction (Fig. 3, lane 1). However, after induction with IPTG muscle FABP was

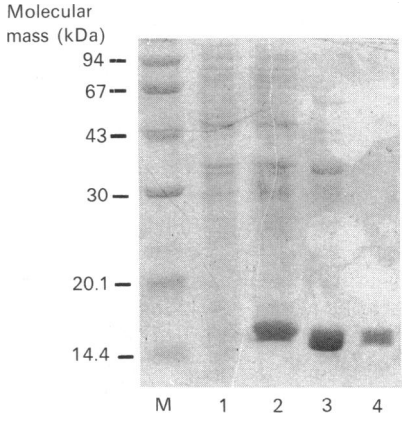

Fig. 2. SDS/PAGE pattern of the purification procedure of human muscle FABP produced in *E. coli*

Lane 1, bacterial lysate at an A_{500} of 0.5 before induction; lane 2, bacterial lysate 3 h after induction; lane 3, anion-exchange column eluate; lane 4, purified muscle FABP after Sephadex G-50 gel filtration. The gel contained 11 % polyacrylamide and was stained with Coomassie Brilliant Blue. Lane M, molecular mass markers (kDa): phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soyabean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa).

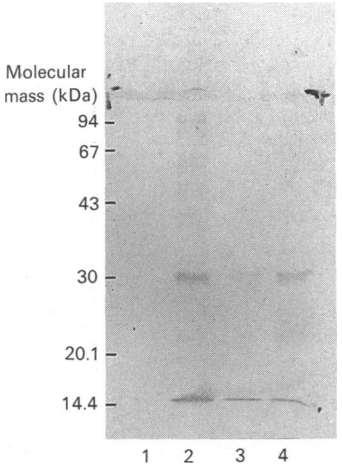

Fig. 3. Immunostained Western blot of muscle FABP produced in *E. coli*

Lane 1, bacterial lysate at an A_{500} of 0.5 before induction; lane 2, bacterial lysate 3 h after induction; lane 3, 5 μg of purified muscle FABP after Sephadex G-50 gel filtration; lane 4, 5 μg of muscle FABP freshly isolated from human skeletal muscle. The blot was stained with anti-(human muscle FABP) antiserum. Molecular mass markers are indicated and are the same as used in Fig. 2.

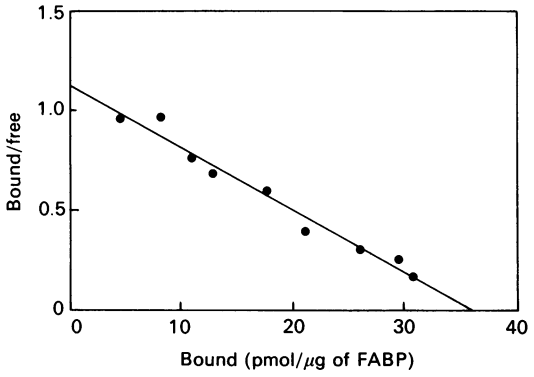

Fig. 4. Scatchard plot analysis of the binding of oleic acid by muscle FABP produced in *E. coli*

Portions of 8 μg of muscle FABP were incubated with 0.1–3.0 μM-[1-^{14}C]oleic acid in a total volume of 0.5 ml of 10 mM-Tris/HCl (pH 8.0). After equilibration, protein-bound and unbound fatty acid were separated by the use of Lipidex at 0 °C.

recognized in the total bacterial lysate (Fig. 3, lane 2) and in the freshly isolated pure form (Fig. 3, lane 3) by the anti-(human muscle FABP) antiserum. The 30 kDa band, which is recognized by the antiserum in the bacterial lysate after induction (Fig. 3, lane 2), must be an aggregate of the muscle FABP, because before induction this protein band was not present (Fig. 3, lane 1). Another indication for the existence of an aggregate is that the 30 kDa compound also appears in the originally pure 15 kDa protein preparation from human psoas muscle after storage at −80 °C (Fig. 3, lane 4).

Binding properties of muscle FABP from *E. coli*

The K_d for oleic acid was obtained from Scatchard plot analyses of binding data (Fig. 4) and was estimated to be 0.58 ± 0.13 μM ($n = 5$). The binding stoichiometry obtained from these data resulted in binding of 0.72 ± 0.09 mol of fatty acid/mol of FABP. In a 1:1 competition assay of radiolabelled oleic acid against various competitors, the preference for ligand binding of muscle FABP was tested (Table 1). From the results it is clear

Table 1. Displacement by fatty acids of [1-^{14}C]oleic acid from muscle FABP derived from *E. coli*

Displacement is given as a percentage of [1-^{14}C]oleic acid binding to muscle FABP. Concentrations were 0.67 μM muscle FABP, 1.0 μM-[1-^{14}C]oleic acid and 1.0 μM-fatty acid. Results are means ± S.D. of 3–5 experiments.

Fatty acid	Displacement (%)
Lauric acid ($C_{12:0}$)	5±2
Myristic acid ($C_{14:0}$)	5±2
Palmitic acid ($C_{16:0}$)	16±3
Stearic acid ($C_{18:0}$)	4±3
Nonadecanoic acid ($C_{19:0}$)	0
Oleic acid ($C_{18:1}$)	33±4
Linoleic acid ($C_{18:2}$)	45±5
Arachidonic acid ($C_{20:4}$)	31±3
Erucic acid ($C_{22:1}$)	15±2
11-Dansylaminoundecanoic acid	4±3
1-Pyrenedodecanoic acid	2±1
2-Bromopalmitic acid	20±3
2-Hydroxypalmitic acid	0

that muscle FABP has a preference for binding to palmitic acid and to C_{18}–C_{22} (poly)unsaturated fatty acids. A marked affinity was also observed for 2-bromopalmitic acid, but not for 2-hydroxypalmitic acid. A slight or no competition for binding of oleic acid to FABP was observed with lauric acid, myristic acid, stearic acid, nonadecanoic acid, 11-dansylaminoundecanoic acid and 1-pyrenedodecanoic acid. Palmitoyl-CoA, palmitoylcarnitine and hydrophobic ligands such as eicosanol, prostaglandins (A_1, E_1, E_2 and $F_{2\alpha}$), thromboxane B_2, retinol, retinoic acid, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate and tetradecylglycidic acid could not displace oleic acid binding at a 1:1 ratio.

DISCUSSION

The use of the pET8c expression vector has the advantage over other expression systems in *E. coli* [13–16,18] in that the T7 RNA polymerase recognizes different promoters and elongates chains about five times faster than the *E. coli* RNA polymerase [28].

Human muscle FABP is efficiently expressed in *E. coli* and the yield of muscle FABP as a proportion of total cellular protein is of the same order or up to 12-fold greater than in other expression systems described previously [14,16–18]. The isolation procedure was adapted from the procedure described by Lowe *et al.* [13] by using a sonifier and an anion-exchange column instead of a French press and Zeta-prep cartridge. The physicochemical data for the *E. coli*-derived muscle FABP are similar to those for FABP isolated from human skeletal muscle [9].

The dissociation constant for oleic acid is in the same range as for the isolated muscle preparation [9]. This value is lower than the values obtained for rat intestinal and liver FABP produced in *E. coli* for palmitic, oleic and arachidonic acids [13], but in the range (0.2–1 μM) of the dissociation constants for oleic acid of all FABP types isolated from tissues [1–3,23,31]. The binding data indicate a binding stoichiometry of 1 mol of fatty acid/mol of protein, which is in agreement with the tissue preparation [9]. The competition assays demonstrated that muscle FABP only binds palmitic acid and long-chain (poly)unsaturated fatty acids, and no other hydrophobic ligands, in contrast with liver FABPs [1–3,23,29,30]. No binding was observed of 1-pyrenedodecanoic acid, whereas liver FABPs and pig and rat heart FABP have a comparable affinity for this fatty acid as for oleic acid [23]. Dansylaminoundecanoic acid was less efficiently bound by these

heart FABP preparations [23]. We observed no binding of palmitoyl-CoA or palmitoylcarnitine, as demonstrated for human heart FABP [31,32]. The acyl-CoA binding observed for heart FABP is probably partially due to contamination with acyl-CoA-binding protein [33]. Therefore the specific function of the muscle FABP type seems to concern the binding and transport of fatty acids to the mitochondria or peroxisomes for β-oxidation.

The aggregation observed for muscle FABP during storage and on expression in *E. coli* was recently also described for human heart FABP by Nielsen *et al.* [34]. They demonstrated that the single thiol group of this protein was involved in the aggregation. The renal FABP type also showed this behaviour [6].

The expression of muscle FABP in *E. coli* offers the possibility of obtaining sufficient protein for crystallization and Röntgen diffraction analysis of its tertiary structure and for n.m.r. analysis of the fatty-acid–protein interaction. Site-directed mutagenesis in the fatty-acid-binding domain is now possible for the study of the binding and transfer activity of this protein.

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