Isolation, Characterization, and cDNA Sequence of Two Fatty Acid-binding Proteins from the Midgut of Manduca sexta Larvae*

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Two abundant fatty acid-binding proteins (MFB1 and MFB2) were isolated from the midgut cytosol of larval Manduca sexta. As isolated, MFB1 and MFB2 were found to contain bound fatty acids in a 1:1 molar stoichiometric ratio. Immunological screening demonstrated that MFB1 and MFB2 were restricted to the midgut in a gradient distribution, with MFB1 more concentrated in the anterior two-thirds of the midgut and MFB2 more concentrated in the posterior two-thirds of the midgut. MFB1 exchanged fatty acid more readily than did MFB2. MFB1 was about 2% and MFB2 about 12% of the cytosolic protein in the midgut.

cDNA clones for MFB1 and MFB2 both encode proteins of 131 amino acids that are rich in lysine and acidic residues. Analysis of the amino acid sequence alignment of the MFBs with six mammalian fatty acid-binding proteins revealed a number of shared features: 9 conserved glycines, presumably important in turns of the β-strands; a basic amino acid in a position corresponding to the residue reported to participate in binding the carboxyl group of the fatty acid (Arg in MFB1 and Lys in MFB2); and conservation of many of the residues important in binding the aliphatic portion of the fatty acid.

Fatty acid-binding proteins (FABPs) are low molecular mass proteins (14–17 kDa), which are members of a superfamily of cytoplasmic hydrophobic ligand-binding proteins (Sweetser et al., 1987). Until recently, the only well characterized proteins belonging to this family have been isolated from vertebrate tissues and consist of the following proteins: heart, liver, renal, and intestinal FABP; cellular retinol-binding protein I and cellular retinol-binding protein II; cellular retinoic acid-binding protein; the P2 protein of peripheral myelin; the p422 (aP2) adipocyte protein; bovine mammary-derived growth inhibitor; and gastrotropin (Veerkamp et al., 1991). The best studied of these proteins are the FABPs for which a number of roles have been proposed: facilitating fatty acid uptake by cells, targeting fatty acids to organelles and specific pathways, altering the activity of enzymes involved in fatty acid metabolism, or protecting cellular proteins and membranes from the detergent effects of fatty acids or their CoA derivatives (Ockner, 1990). In spite of considerable effort, the physiological role(s) played by these proteins remains obscure.

FABP has been recently identified in the flight muscle of the migratory locust, (Haunerland and Chisholm, 1990), and a cDNA clone from the blood fluke Schistosoma mansoni was found to encode a FABP (Moser et al., 1991). Because it processes large amounts of lipid, we investigated the possibility that the larval midgut of Manduca sexta might also contain FABP. In this paper we report the purification and characterization of two FABPs (MFB1, MFB2) and their cDNA sequences from the midgut of the larval tobacco hornworm, M. sexta.

MATERIALS AND METHODS

M. sexta were raised on a high wheat germ diet as previously described (Prasad et al., 1986; Fernandez-Warnakulasuriya et al., 1988). Benzamidine, and phenylmethylsulfonyl fluoride (PMSF) were from Aldrich. We obtained Sephadex G-75, Sephacryl S-300 HR, and high molecular weight standards for calibration of gel filtration columns from Pharmacia LKB Biotechnology, Inc.; DEAE-Trisacryl M from IBF Biotechnics (Villeneuve-la-Garenne, France); and Coomassie Brilliant Blue R-250 from Pierce Chemical Co. [14C]Oleic acid was from Du Pont-New England Nuclear.

Purification of Fatty Acid-binding Proteins—Midgut tissue from day 2 fifth instar larvae was homogenized with 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5 mM PMSF, and 5 mM benzamidine (5 mg/6 of tissue) in a polytron homogenizer at setting 6 for 20 s (Brinkmann Instruments, Inc., Westbury, NY), and the homogenate was centrifuged at 100,000 × g for 60 min in a Ti-60 fixed angle rotor. In an ice bath, the 100,000 × g supernatant was adjusted to 75% saturation in ammonium sulfate and the suspension was centrifuged (10,000 × g for 30 min). The supernatant was adjusted to 95% saturation in ammonium sulfate; the precipitate was collected by centrifugation, dialyzed against ammonium sulfate, and the volume was reduced to 5 ml/g of tissue, using a Diaflo Ultrafiltration Membrane, YM-10 (Amicon Corp., Danvers, MA). During the development of the purification scheme, FABP was followed by labeling it with radioactive fatty acid. This was accomplished by adding 5 μCi of [14C]potassium oleate to the solution containing the dialyzed, resuspended 95% ammonium sulfate pellet and mixing for 4 h at 4 °C in a tube rotator. For large scale purifications, fractions were assayed for FABP using a fatty acid binding assay (see below). It should be noted that these procedures only detected MFB1. MFB2 was found when the low molecular weight proteins from midgut were analyzed for fatty acid.

The solution from the ammonium sulfate step was applied to a Sephadex G-75 Superfine column (2.5 × 110 cm) equilibrated in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5 mM PMSF, and 5 mM benzamidine and eluted with the same buffer at a flow rate of 11.5 ml/h and collected in 2.5-ml fractions. The fractions were assayed for protein using the BCA method (Smith et al., 1985), and for fatty acid binding; selected fractions were subjected to SDS-PAGE. The
low molecular weight fractions testing positive for fatty acids were pooled and applied to a DEAE-Trisacryl M column (2.5 × 8 cm) packed in the same buffer used for gel filtration, but without NaCl. Protein was eluted with a linear NaCl gradient (0–100 mM) in the same buffer at a flow rate of 35 ml/h, and 1.6-ml fractions were collected. The fractions were assayed for protein, fatty acid binding, and selected fractions were subjected to SDS-PAGE. In addition to fractions containing impure MFB1, the DEAE column gave pure preparations of two other low molecular mass proteins, which had molecular masses on SDS-PAGE of 15 and 17 kDa, respectively. As shown below, the 15-kDa protein was also a fatty acid-binding protein (MFB2).

The fractions containing MFB1 were pooled and dialyzed against 25 mM Imidazole buffer, pH 7.4, and applied to a PBE 96 chromatofocusing column (Pharmacia LKB Biotechnology Inc.) and eluted with pH 4.0 polybuffer/water 1% (v/v) according to the manufacturer’s instructions. Fractions were assayed for protein, fatty acid binding, and pH. The fractions containing pure MFB1 were concentrated by ammonium sulfate precipitation as described above.

**Fatty Acid Binding Assay**—Fatty acid binding was measured by a modification of the method of Morrow and Martin (1985). The sample containing FABP and varying amounts of [14C]potassium oleate (11.6 μCi/μmol) in 200 μl of 10 mM Tris, pH 7.5, containing 150 mM NaCl, was incubated for 1 h at 25 °C. In order to remove unbound fatty acid, 50 μl of a 2% suspension of charcoal (Norit A) in 0.2% Dextran T70 was added and the sample was centrifuged in a microfuge for 5 min; the supernatant was assayed for protein and radioactivity. Control experiments showed that 97% of added FABP was recovered in the supernatant and only 1% of labeled fatty acid remained in the supernatant. In some experiments the time of incubation or pH were varied as detailed in the figure legends.

**Immunology**—Antibodies for immunoblotting were raised by injecting the protein-adjuvant mixture (Ribi Immunochem Research, Hamilton, MT) into the breast muscle of hens. IgY was purified from egg yolk using the method of Polson et al. (1985). SDS-PAGE-separated proteins were electrophoretically transferred to nitrocellulose and immunoblotted according to the method of Burnette (1981) using IgY and rabbit anti-chicken IgY coupled to horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA).

Antiserum for cDNA library screening was produced in New Zealand White rabbits by intramuscular injection of the protein-adjuvant mixture (Ribi Immunochem Research, Hamilton, MT). The serum was stored at −70 °C.

**Tissue Distribution**—The following samples were used: whole midgut, midgut divided into three sections (anterior to posterior), fat body and muscle from day 2 fifth instar larvae, and eggs from day 2 adults. For each sample a 100,000 g supernatant was prepared as described above, and 100 μg of protein was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted.

**Electrophoresis**—SDS-polyacrylamide gel electrophoresis was carried out using the method of Laemmli (1970) in slab gels containing either 15% or a 4-15% linear gradient of polyacrylamide. Gels were stained with Coomasie Brilliant Blue R-250.

**Lipid Analysis**—The fatty acid content of proteins was determined as previously described (Fernando-Warnakulasuriya et al., 1981), using 5 mg of protein.

**RNA Isolation**—Total RNA was prepared from the midgut of a day 2 fifth instar male larva using the procedure of Savakis et al. (1986). Polyadenylated RNA was selected by two passages through an oligo(dT)-cellulose column according to the method of Aviv and Leder (1972).

**cDNA Library Construction and Screening**—Five μg of each polyadenylated RNA was used to prepare cDNA and a directional library from a commercial kit (ZAP-cDNA Synthesis Kit; Stratagene, La Jolla, CA). The cDNA library was screened with rabbit antisera using goat antirabbit IgG and an alkaline phosphatase color development system (Bio-Rad).

**DNA Sequencing**—Both MFB clones were subcloned into pBluescript SK+ plasmid using an in vivo excision protocol (Stratagene, La Jolla, CA). Both single and double-stranded DNA were sequenced by the dyeoxy chain termination method (Sanger et al., 1977).

**Sequence Alignments**—Protein sequences were aligned using the multiple sequence alignment procedure of Feng and Doolittle (1987).
### Table I

**Summary of the purification of MFBl from M. sexta larval midgut**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total activitya</th>
<th>Specific activityb</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000 X g supernatant†</td>
<td>4669</td>
<td>5329 (100%)</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>75-95% ammonium sulfate fraction</td>
<td>952</td>
<td>3840 (72.2%)</td>
<td>4.0</td>
<td>3.6</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>253</td>
<td>2660 (48.9%)</td>
<td>10.3</td>
<td>9.4</td>
</tr>
<tr>
<td>DEAE-trisacryl M</td>
<td>89.9</td>
<td>2260 (42.5%)</td>
<td>25.1</td>
<td>22.8</td>
</tr>
<tr>
<td>Chromatofocusing</td>
<td>32.8</td>
<td>1900 (35.7%)</td>
<td>57.9</td>
<td>52.6</td>
</tr>
</tbody>
</table>

† Total nanomoles of fatty acid bound in fatty acid binding assay. Percent recovery in parentheses.
‡ Nanomoles of fatty acid bound/mg protein.
§ From 267 midguts.

### Table II

**Fatty acid content and composition of midgut fatty acid-binding proteins**

Five mg of MFBl (0.34 µmol) and MFB2 (0.35 µmol) were quantitatively analyzed for fatty acid content and composition. The data are means ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>MFBl mol fatty acid/mol protein</th>
<th>MFB2 mol fatty acid/mol protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>0.17 ± 0.05</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>16:1</td>
<td>0.04 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>18:0</td>
<td>0.04 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>18:1</td>
<td>0.10 ± 0.02</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>18:2</td>
<td>0.39 ± 0.06</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td>18:3</td>
<td>0.09 ± 0.03</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Total</td>
<td>0.83 ± 0.05</td>
<td>0.90 ± 0.04</td>
</tr>
</tbody>
</table>

### Results and Discussion

#### Isolation and Characterization FABPs—Figs. 1–3 illustrate the purification of MFBl in which the purification was followed using [14C]oleic acid to label the protein. Table I presents a summary of the large scale purification in which MFBl was followed using a fatty acid binding assay. These quantitative data, with a recovery of 35% and a purification of 50-fold, show that MFBl comprises approximately 2% of the soluble protein of the midgut tissue.

The elution profile presented in Fig. 2 shows that, in addition to MFBl there were two other low molecular weight proteins in the midgut supernatant with molecular masses of 17 and 15 kDa. The latter protein (MFB2) is clearly a major protein in the midgut and appears to bind a small amount of [14C]oleic acid (see Fig. 2). In the large scale preparation detailed in Table I, we isolated 250 mg of MFB2. By assuming a recovery similar to MFBl from the DEAE column, MFB2 comprises about 12% of the soluble protein in the midgut. In the same preparation we recovered 14.5 mg of the 17-kDa protein. The fact that the MFBs are major components of the cytosolic proteins is consistent with that reported for mammal FABPs (Veerkamp et al., 1991).

In order to further characterize these three proteins, we measured fatty acid and other lipid content. MFBl and MFB2 both contained 1 mol of free fatty acid/mol of protein, but the 17-kDa protein contained negligible fatty acid (Table II). A 1:1 molar ratio of fatty acid to protein is in accordance with that reported for the vertebrate and locust FABPs (Veerkamp et al., 1991; Haunerland and Chisholm, 1990). The two MFBs have essentially the same fatty acid composition, which corresponds closely to the fatty acid composition of the artificial diet (Fernando-Warnakulasuriya et al., 1988). Neither MFBl nor MFB2 contained any lipids other than fatty acids when the lipid extracts were examined by thin layer chromatography (data not shown). In general fatty acids predominate in vertebrate FABPs, with the liver and kidney forms being the exceptions (Veerkamp et al., 1991).

Fig. 4 compares the binding of [14C]oleic acid to MFBl and MFB2. These data show that MFBl has a single high affinity binding site with an apparent dissociation constant of about 14 µM. It should be kept in mind that we are measuring exchange of fatty acid, not true dissociation constants in these experiments, since the protein already has a bound fatty acid when isolated. This is quite apparent in analyzing the data for MFB2. We were never able to saturate the protein with labeled fatty acid and the estimated dissociation constant was greater than 100 µM. These data initially lead us to believe...
WFB2 SYLCKVYSLVKOEYFDGFLKSAGLSDDKlOALVSDKPTOKWEANGDSYSlTSTGLGGERTVSFKSGVE
WFB1 AYLCKVYKFDREENDGFLKSIGLSEEOVOKYLOYKPSOLVKEGDKYKYlSVS~GTKETVFSGVE
WFB2 TDDWOGGLPIKTTYTMG  NTVTOWN SAOGSAlFKREYNGDELKVTlTSSEWGVAYRYYKA
HFBL SFSGK YOLOSOEYFEAFWKAlGLPEELlOKGKDlKGVSElVONGKHFKFTlTAGSKVlONEFTVGEE
RFBI fAVSLADGTELTGTLTWEG YKLVGKFKRWNGYELIAVREISGNELIOTYTYE GVEAKRIFKK
HFBL CELETWTCEKVKAWDIEGDNKWVTTFK GIKSVTEFNGDTITNTWTLG  DIVYKRVSGRI
HFBl  fNYNLMGTELRGTUSLEC NKL1GKFKRTDNGNELNTVREllCOELVOTYVYE CVEAKRIFKKD
HFBL CELETMTGEKVKTWOLEGDNKLVTTFK YIKSVTELNGDIITWTMTLG DlVFKRlSKRl
FIG. 6. Partial restriction map, sequencing strategy, and nucleotide and deduced amino acid sequence of MFB1 (top) and MFB2 (bottom).

The occurrence of two FABPs within a single organ has

that MFB2 was not a fatty acid-binding protein. Since these experiments measure exchange only under one set of conditions, one must be cautious about interpreting the apparent dissociation constants. In fact, the apparent dissociation constants are too high to be consistent with the fact that both FABPs are isolated with 1 mol of bound fatty acid.

Fig. 5 presents data on the tissue-specific distribution of MFB1 and MFB2. The FABPs were found only in the midgut and not in the fat body, muscle, or eggs. Interestingly, MFB1 was found predominately in the anterior two-thirds of the midgut, whereas MFB2 was found only in the posterior two-thirds of the midgut. The absence of MFB2 from the anterior one-third of the midgut is evident even in the Coomassie-stained gel. Hence MFB1 and MFB2 are similar to the mammalian liver and intestine FABPs which exhibit a tissue distribution gradient (Glatz and Vusse, 1990). The 17-kDa protein was found in the midgut, where it had a distribution similar to that for MFB2, and in small amounts in the egg.

cDNA Cloning—From 30,000 recombinant plaques, 23 MFB1 and 150 MFB2 positive cDNA clones were identified by immunological screening. One positive clone from each of the two screenings was purified, subcloned into pBluescript SK+ plasmid, restriction mapped, and sequenced (Fig. 6). The cDNAs were of similar size (491 and 465 bases for MFB1 and MFB2, respectively) and encoded proteins of 131 amino acid residues (Fig. 6). The deduced amino acid composition revealed that both proteins are rich in acidic amino acids and lysine with molecular masses of 14.7 and 14.1 kDa (MFB1 and MFB2, respectively), not unlike the vertebrate FABPs (Maatman, 1991). The cDNA for MFB1 contains an open reading frame beginning with an ATG codon at position 12 and extending to position 407, followed by an 84-base pair 3'-untranslated sequence (Fig. 6). The open reading frame for the MFB2 cDNA begins with an ATG codon at position 6 with the translated region extending to position 401 and a 64-base pair 3'-untranslated region (Fig. 6). All FABPs isolated to date have an acetyl group at the NH2-terminal amino acid (Bernier and Jollé, 1987). Several attempts to sequence the MFBs by Edman degradation were unsuccessful, suggesting that the NH2 terminus of these proteins may also be acetylated.

Analysis of the progressive amino acid alignment of the MFBs with six mammalian FABPs by the method of Feng and Doolittle (1987) revealed a number of shared features (Fig. 7). The two MFBs are 55.7% identical in amino acid sequence. Whereas the percent amino acid identities between mammalian FABPs from the same tissues of different species are high, ranging from 80 to 90%, mammalian FABPs from different tissues are about as similar to each other (24-33.3%) as they are to the MFBs (20.3-33.3%). Similar conclusions were reached from analysis of the pairwise alignment of cDNA sequences (see table in Fig. 7).
been observed in vertebrate tissues: liver and intestine FABP of rats and humans are both found in the intestine (for review, see Veerkamp et al., 1991) and two types are reported in mammalian kidneys (Maatman et al., 1991; Lam et al., 1988). However, the percent amino acid identity between the liver and intestine forms is considerably lower (28.2–24.8%) than the similarity between the two MFBs (55.7%).

Elucidation of the three-dimensional structure of rat intestinal FABP through x-ray crystallography (Sacchettini et al., 1990) permits the identification of several important conserved residues among the FABPs. Rat intestinal FABP consists of two orthogonally oriented β-sheets with glycines facilitating turns between β-strands. These 7 glycines are conserved in the MFBs (Fig. 7). Also highly conserved are the residues involved in binding of the fatty acid. An arginine participates in the electrostatic interaction with the carboxyl group of the fatty acid (Sacchettini et al., 1990). This residue is conserved in MFB1 and is substituted with another basic amino acid, lysine, in MFB2. This change may account for the high affinity of MFB2 for fatty acids. We are currently investigating this phenomenon. It is interesting to note that the basic amino acid (i.e. arginine) purported to act in the fatty acid carboxyl group is not conserved in the liver FABPs where the carboxyl group is reported to interact near the aqueous surface of the protein (Cistola et al., 1989). Moreover, of the 18 residues associated with the binding of the fatty acid hydrocarbon chain, 10 and 12 residues of MFB1 and MFB2, respectively, are either retained or conservatively substituted.

Based on the physiochemical and cDNA data, we conclude that MFB1 and MFB2 are fatty acid-binding proteins. The MFBs have molecular weights, amino acid residue numbers and composition, and lipid composition in agreement with those described for the vertebrate and locust FABPs (Ockner, 1990; Haunerland and Chisholm, 1990). Amino acid sequence alignments (Fig. 7) suggest that the three-dimensional structure of the MFBs and the FABP may be similar. Presently x-ray crystallographic studies are being conducted which will elucidate the structure of MFB2.

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REFERENCES