Fatty Acid Binding Protein

ISOLATION FROM RAT LIVER, CHARACTERIZATION, AND IMMUNOCHEMICAL QUANTIFICATION*

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Fatty acid-binding protein (FABP) was identified and isolated from rat liver cytosol by gel filtration, thin layer isoelectric focusing, and affinity chromatography. FABP (M, 12,080 ± 80) exists in several immunochromically identical forms differing in isoelectric pH, which may in part reflect differences in their respective complements of bound endogenous ligand. FABP-bound fatty acids accounted for 60% of total cytosolic long chain fatty acids but contained no detectable phospholipid; the substantial enrichment of FABP in 18:2 and 20:4 as compared with whole liver homogenate was not influenced by homogenization of tissue in EDTA. The amino acid composition of FABP suggests that it is closely related or identical with certain similar neutral and acidic cytosolic proteins reported from other laboratories. By quantitative radial immunodiffusion, FABP concentration in cytosol from livers of sexually mature male rats exceeded that from mature males (51.7 ± 5.0 vs 39.8 ± 4.0 μg/mg of protein, p < 0.05), confirming earlier studies in which sex steroid effects on rates of fatty acid utilization were correlated with FABP concentration as determined by means of a binding assay. The abundance of FABP, its importance in the cytosolic binding of endogenous as well as exogenous fatty acids, and its demonstrated correlation with rates of hepatocyte fatty acid utilization provide additional evidence for its relationship to the cellular metabolism of long chain fatty acids.

EXPERIMENTAL PROCEDURES

Materials—Sephadex G-75, G-50, and G-25 were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J., Affi-Gel 102, acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine, ammonium persulfate, bromphenol blue, and Coomassie brilliant blue R-250 were obtained from Bio-Rad. Amphoteric carrier ampholytes were obtained from LKB Instruments, Inc., Stockholm, Sweden. Ethanolamine and riboflavin were obtained from Eastman Kodak Co., Rochester, N.Y. Sodium oleate, carbodiimide (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide HCl), Tris (2-amino-2-hydroxymethyl-1,3-propanediol) base, sodium dodecyl sulfate, and 2-mercaptoethanol were obtained from Sigma; complete Freund’s adjuvant was purchased from Grand Island Biological Company, Grand Island, N.Y.; 1-anilino-8-naphthalene sulfonic acid, magnesium salt, was purchased from Pierce Chemical Co.; acetic anhydride was purchased from MCB Manufacturing Chemists, Cincinnati, OH. YM-5 membranes used in concentration of protein solutions were obtained from Amicon Corporation, Lexington, MA. [1-14C]Oleic acid was obtained from New England Nuclear.

Preparation of Rat Liver Cytosol—Livers obtained from fed adult male Sprague-Dawley rats, 300 to 400 g, were perfused with 0.85% NaCl at 4 °C, homogenized in 0.154 M KCl -0.01 M PO buffer (pH 7.4, 2 ml/g), and centrifuged for 20 min at 12,000 × g, and the resulting supernatant was centrifuged for 1 h at 105,000 × g (5, 6, 14). The clear high speed supernatant fraction was aspirated so as to minimize contamination by floating fat and was used as such for subsequent gel filtration or binding experiments or, after inlaced modifications, for other analytical or preparative procedures. For resolution of cytosolic...
protein fractions and/or analysis of fatty acid binding thereto, cytosol, or the indicated cytosolic fraction was mixed with [14C]oleate in 5 μl of propylene glycol prior to subsequent separation by gel filtration. In some experiments, whole cytosol or fractions thereof were delipidated by extraction for 30 min with 2 volumes of di-isopropyl ether (15); the phases were separated, and a second extraction was done for 10 min with 1 volume of di-isopropyl ether. After centrifugation, the aqueous phase was obtained, and residual di-isopropyl ether was removed under vacuum at 37 °C for 10 min or by lyophilization.

Lipid Analysis—Lipids were extracted from native or delipidated samples by the method of Folch et al. (16) and were separated by thin layer chromatography (0.25 mm Silica Gel 60 in petroleum ether:ethyl ether:glacial acetic acid, 90:15:1). Appropriate zones were identified by means of standards and were extracted from the chromatogram. Fatty acid methyl esters were prepared by the addition of diazomethane to the fatty acid fraction and were analyzed as previously described (14) by gas-liquid chromatography on a Hewlett-Packard 402 B gas chromatograph employing a 5-foot glass column packed with 10% SP-2230 on 100/120 mesh Chromosorb W AW (Supelco) at 186 °C. Pentadecanoic acid was employed as an internal standard; fatty acid mass was determined by means of a Hewlett-Packard model 3380 digital integrator. Phospholipid was measured in these experiments by the method of Barlett (17) as modified by Marias et al. (18).

Electrophoresis and Isoelectric Focusing—Polyacrylamide disc gel electrophoresis (19) employed 3.5% concentrating (pH 6.4) and 7% separating (pH 8.9) gels buffered with 0.5 M Tris and 0.38 M glycine (pH 8.6, 20°C). 3 mA/gel for 120 min. Gels were fixed and stained with 1% Amido schwarz in 7% acetic acid or 0.2% Coomasie brilliant blue in methanol-water-acetic acid (5:5:1) and were destained electrophoretically. In some experiments, gels were exposed to 0.035% 1-anilino-8-naphthalene sulfonic acid, magnesium salt, in 0.1 M NaPO4 buffer (pH 6.8) and were examined under ultraviolet light (20). Fluorescent protein bands corresponding to the appropriate zones identified by fixation and staining were sliced from the gel, homogenized, and either extracted repeatedly with KCl-phosphate buffer or a solution of 6 M urea, 1 mM EDTA, and 1 mM dithiothreitol in 0.05 M glycine pH 10.54, or used directly for preparation of antisera.

Isoelectric focusing of the M, 12,000 FABP fraction was carried out on a thin layer of Sephadex G-75 superfine added to a solution of 11.4 mM lysine and 9.6 mM arginine in 30 ml of H2O to which was added 4 ml of LKB ampholytes (pH 3.5 to 10) and 6 ml of sample protein in 0.13 M glycine (21); correspondingly higher volumes were used in preparative runs. In some experiments, [14C]methyl oleate was added to the protein sample so as to provide an electrically neutral marker of long chain acyl-binding fractions. Samples were subject to focusing at 400 V (4 °C) and allowed to stabilize for at least 18 h. Tracings of the focused gels were made by brief application of a strip of special print paper (Whatman no. 1) to the gel. After centration, the aqueous phase was obtained immediately through or which were bound and subsequently eluted with 25% ethanol (pH 6) were analyzed by disc gel electrophoresis.

Affinity Chromatography—This was employed to identify those components of the M, 12,000 FABP fraction which exhibited fatty acid-binding activity. Oleic acid was complexed to Bio-Rad Affi-Gel 101 by the method of Peters et al. (23). The partially purified FABP fraction (5 μl, 10 μg protein) in 0.1 M glycine, pH 10, was applied to the column. After centrifugation, the aqueous phase was obtained immediately through or which were bound and subsequently eluted with 25% ethanol (pH 6) were analyzed by disc gel electrophoresis.

Immunological Studies—Protein fractions (approximately 200 μg) separated by polyacrylamide gel electrophoresis or thin layer isoelectric focusing were mixed with an equal volume of complete Freund's adjuvant and administered in multiple intrastridial sites to New Zealand white rabbits. Specificity of antisera and immunochromatography of antibodies in various protein fractions were assessed by the Ouchterlony double immunodiffusion method (24).

RESULTS

Identification of Principal Fatty Acid Binding Protein Fraction in Rat Liver Cytosol—Although it has been well documented that exogenous 14C-labeled long chain fatty acids are almost entirely bound to one or more proteins in the M, 12,000 range, the distribution of endogenous long chain fatty acids among cytosolic protein fractions has not been defined. To address this question, whole rat liver cytosol was fractionated by Sephadex G-75 gel filtration; fractions were analyzed separately for protein (absorbance, 280 nm) and were combined into four larger fractions for analysis of fatty acid and Lowry protein content (Fig. 1). It can be seen that fractions 30–34, the midpoint of which approximated the elution volume of M, 12,000 globular proteins, contained 60.3% of cytosolic long chain fatty acids (13.5 nmol of fatty acid/mg of protein) and far exceeded the other fractions in the amount of fatty acid per mg of protein. Moreover, even further enrichment was observed in the M, 12,000 fraction purified by two consecutive gel filtrations through Sephadex G-50 (Table I). The FABP correction of the Lowry determination (see “Experimental Procedures”) was not applied to experiments involving whole cytosol or M, 12,000 fraction; its application to the M, 12,000 fraction in Fig. 1 and Table I would have increased even further the differences in fatty acid:protein ratio between FABP and other fractions of cytosol.

Homogenization of liver in a buffer containing 5 mM EDTA did not significantly affect the fatty acid composition of the FABP fraction, suggesting that its apparent enrichment in endogenous fatty acid fractions did not reflect artificial phospholipase A ω-mediated fatty acid release during preparation of the sample. Furthermore, this fraction was found to contain virtually no detectable phospholipid (i.e. less than 0.01 amol/mg of protein), suggesting that it is not likely to be involved

1 R. K. Ockner, J. A. Manning, and J. P. Kane, unpublished observations.
to a substantial extent in phospholipid transfer or exchange. Delipidation of the FABP fraction with di-isopropyl ether, as described under "Experimental Procedures," removed virtually all of these noncovalently bound long chain fatty acids (Table I). Following delipidation, the protein in this fraction was stable for several weeks.

Isolation of FABP—The partially purified FABP (M, 12,000) fraction obtained by Sephadex G-50 gel filtration of rat liver cytosol was subjected to thin layer isoelectric focusing in the presence of tracer $[^{14}C]$methyloleate to identify fractions with significant affinity for long acyl chains. Native and delipidated preparations were analyzed similarly in simultaneous with significant affinity for long acyl chains. Native and delipidated preparations were resolved into a large number of components. For both preparations, however, recovered radioactivity was principally in the presence of tracer $[^{14}C]$methyloleate and/or may reflect con-

FIG. 2. Thin layer isoelectric focusing of $[^{14}C]$methyloleate with the FABP (M, 12,000) fraction of rat liver cytosol. Native and delipidated FABP fraction (16 mg) partially purified from whole cytosol by Sephadex G-50 gel filtration and $0.64 \text{ nmol of } [^{14}C]\text{methy}-$

Endogenous fatty acid composition of M, 12,000 FABP fraction from rat liver cytosol

The FABP fraction (M, 12,000) was partially purified from rat liver cytosol by gel filtration twice in succession on Sephadex G-50 and was extracted and analyzed for free fatty acid by gas-liquid chromatography (see "Experimental Procedures"). The "EDTA" sample was prepared after homogenization of tissue in 5 mM EDTA. The "delipidated" sample was extracted (after partial purification) with diisopropyl ether (see "Experimental Procedures").

| 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:4 | Total |
|------|------|------|------|------|------|-------|
| Native ($n = 3$) | 7.17 ± 0.12 | 2.92 ± 0.14 | 4.17 ± 0.55 | 3.63 ± 0.08 | 0.07 ± 0.04 | 3.66 ± 0.48 | 21.62 ± 0.44 |
| EDTA | 6.38 | 3.38 | 3.59 | 3.34 | 3.31 | 20.00 |
| Delipidated | 0.32 | 0.10 | 0.10 | 0.34 | 3.31 | 0.52 |
proteins in the focusing gel and which did not bind significantly in these experiments did not enter the disc gel, as would be expected.

The material from the lower band on disc gel (corresponding to Band 2B in isoelectric focusing) was employed as an antigen for preparation of an antiserum to FABP. This antiserum produced a single line of immunoprecipitation against the partially purified M, 12,000 fraction (Fig. 4, A and B, top and bottom wells). It can be seen that there were reactions of identity between this line and that formed with Bands 2A, 2B, 3, 4, and 5. The same pattern was observed with antiserum prepared against Band 5. The very weak reaction of Band 1 (Fig. 4A) reflects contamination of this fraction by one of the acidic immunochemically identical forms of FABP as shown on overloaded gels. Band 6 (not shown) also showed a reaction of identity, whereas the more basic fractions separated by isoelectric focusing failed to react with the antiserum. Of significance, the major FABP bands (Figs. 2 and 3) thus share immunochemical identity but differ from one another in charge and in isoelectric pH; this may in part reflect the amount of bound endogenous long chain fatty acid (as suggested by the effect of delipidation in Fig. 2) or differential binding of ampholines (30). Such an influence of noncovalently bound ligand on charge behavior could account for the observation that repeated electrophoresis or isoelectric focusing of an apparently single species results in the “reappearance” of the other major forms of the protein (9).

Other factors which may contribute, e.g. proteolysis or covalent modification, are not excluded, although the failure to observe lower molecular weight forms among the various isoelectric focusing fractions suggests that proteolysis is not likely to be of major significance.

That isoelectric focusing Bands 2-5 represent the principal fatty acid binding components of this material is further demonstrated by affinity chromatography as shown in Fig. 5. In this experiment, the disc gel electrophoretic pattern of the native M, 12,000 fraction is compared with those components which fail to bind to the column and those which did bind and were readily eluted. The latter (i.e. the fatty acid-binding components) consists of 2 major bands and an interposed relatively minor band, corresponding to isoelectric focusing Bands 3-5 (upper) and 2B (lower). The interposed band was inconsistently separable from the upper band and was not further characterized.

**Binding Characteristics of Native and Delipidated Proteins**—To assess the relative binding affinity of the native and delipidated M, 12,000 fractions as well as the isolated FABP (Band 5), a Sephadex G-25 binding assay was employed using [14C]oleate as ligand, as previously described (5). As shown in Table II, the delipidated M, 12,000 fraction exhibited somewhat greater binding of [14C]oleate than did the native material, consistent with the findings on isoelectric focusing (Fig. 2). However, it also can be seen that the binding of [14C]oleate by purified FABP (Band 5) was less than that for the native or delipidated partially purified fraction. This may reflect residual binding of ampholytes to purified FABP after isoelectric focusing, despite the fact that efforts were made to remove them by means of Sephadex G-50 chromatography. The fact that exposure to ampholytes during isoelectric focusing did not appear to substantially diminish binding of [14C]methyl oleate to Band 5 (Fig. 2) may reflect the fact that in this instance protein and lipid were added together prior to their exposure to ampholytes and reduced pH, whereas the isolated Band 5 (Table II) had already been so exposed.

**Molecular Weight of Fatty Acid Binding Protein**—The molecular weight of purified rat liver M, 12,000 fatty acid binding protein (isoelectric focusing Band 5) was found to be 12,080 ± 80 (n = 5) as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In this system, Band 5 regularly migrated as a single band, even on overloaded gels; isoelectric focusing Band 2 occasionally contained much smaller quantities of a larger protein (M, 17,000).

**Age and Sex Differences in Rat Liver FABP Concentration**—Employing the antiserum to purified FABP in a quantitative radial immunodiffusion assay, the concentration of FABP in whole cytosol was determined in livers from sexually immature and mature female and male Sprague-Dawley rats. As shown in Table III, the immature (31-day) females and males did not differ significantly. With maturation, FABP concentration increased in both, but more so in females, so that by the time of sexual maturation (62 days) there was a significantly higher concentration in females than in males. These findings are in essential agreement with earlier measurements of rat liver FABP concentration by means of a binding assay (6). Moreover, the studies indicate that FABP accounts for 3.98 and 5.17% of cytosolic protein in adult male and female rat liver, respectively, and more than 50% of the partially purified M, 12,000 FABP fraction.

**Amino Acid Composition of FABP**—The amino acid composition of FABP (Table IV) showed a balanced distribution of polar and hydrophobic residues, as would be expected in a
A growing body of evidence suggests that the cellular utilization and metabolism of relatively nonpolar poorly water-soluble substances involves the participation of intracellular low molecular weight cytosolic proteins. With certain exceptions these proteins appear not to exhibit enzymatic activity in the usual sense, i.e. in directly catalyzing the formation or cleavage of covalent bonds. Rather, they have been identified largely as the result of, and are principally characterized by, their binding of more or less closely related groups of ligands. These proteins are viewed as facilitating the intracellular transport or exchange of their respective ligands or exerting a permissive or enhancing effect on the entry of these ligands into reactions catalyzed by membrane-bound enzymes (32). Among these proteins are the several sterol carrier (10-12) and phospholipid exchange proteins (13), retinol and retinoic acid-binding proteins (33), the glutathione transferases (the only class of such proteins which in fact appear to be enzymes) (34), the amino azo dye-binding (9) and Z proteins (31), and FABP (1, 5, 6, 14, 35). These proteins in many cases differ substantially in regard to molecular weight, charge, and substrate specificity but share in common the fact that the relationship between their properties in vitro and their function in vivo remains somewhat unclear. Furthermore, it now is evident that a number of these proteins, viewed differently by various investigators in terms of their biological function and significance, and designated accordingly, may in fact be the same (10, 11, 31). The subject of the present communication falls into this category.

The earlier description of FABP as a Mr 12,000 acidic protein is confirmed in the present studies. It is also evident that the isolated protein exists in several immunologically identical and at least partially interconvertible forms, differing in isoelectric pH, and possibly in the amount of bound endogenous ligand. That bound ligand may influence the charge of the carrier molecule is shown by the well documented effect of bound fatty acid on the isoelectric pH of serum albumin (36). Other possible explanations for the existence of charge isomers of FABP are not excluded. Since these different charge forms of the protein are seen as identical by the antiserum employed in these experiments, it has not been

### TABLE II

| Native FABP fraction | 11.1,11.2 |
| Delipidated FABP fraction | 13.2,13.8 |
| Purified FABP (Band 5) | 3.1,4.8 |

### TABLE III

| Age | FABP |
|-----|------|
| | Female | Male |
| days | mg/mg cytosolic protein | 30.1 ± 0.8 | 28.2 ± 0.5 |
| 31 | 31.7 ± 0.3 | 39.8 ± 4.0 |
| 62 |

water-soluble protein. The amino acid composition of FABP is in general agreement with those reported for the “A” and “Z” proteins by Ketterer et al. (9) and Tzulcus and Arias (31), for the squalene and sterol carrier protein by Dempsey et al. (11), and for the “Band C” protein by Billheimer and Gaylor (10). Our analyses for tryptophan using mercaptoethanesulfonic acid, which gives higher yields of tryptophan, confirm the observation of Dempsey et al. (11) that tryptophan is absent from this protein. Our analyses of cysteine gave higher values than those reported for the other proteins. The content of methionine was also appreciably higher in FABP than those reported for the “A” and “Z” protein (9, 31) and the “Band C” protein (10). Because detection of both cysteine and methionine is improved by the performic acid technique, these differences between our analyses and those reported from the other laboratories probably reflect losses of these amino acids in the other analytical techniques.

### DISCUSSION

Amino acid composition of related liver cytosolic proteins

Rat liver cytosolic FABP (Band 5, Fig. 2) was analyzed for amino acid composition as described in “Experimental Procedures.” Values shown for FABP are the means of 6 determinations for each amino acid. Values for A (form II), Band C, sterol carrier protein (SCP), and Z (pH 7.2) are taken from Refs. 9-11 and 31, respectively.
possible to quantify each independent of the others. It is noteworthy, however, that in the aggregate they comprise approximately 5% of liver cytosolic protein and a major portion of the protein mass in the M, 12,000 fraction. Moreover, the important influence of sex and maturation of the animal on the concentration of hepatic FABP as previously demonstrated by binding assay (5, 6) is confirmed immunohistochemically in the present study.

On the basis of the physical properties and the amino acid composition of the protein, it seems highly probable that FABP is in fact the same protein designated by Billheimer and Gaylor (10) and Dempsey (11) as sterol carrier protein, by Ketterer as amino azo dye-binding protein A (9), and by Arias and colleagues as Z (31). These workers have attributed to the protein a role in the metabolism and/or intracellular transport of cholesterol, heme, carcinogens, and cholephilic anions such as bilirubin, sulfobromophthalein, and indocyanine green, respectively, although there is no direct and conclusive indication that any or all of these putative roles is valid in vivo. On the other hand, an increasingly compelling body of circumstantial evidence supports a relationship of this protein to cellular transport and utilization of long chain fatty acids. This evidence can be considered in two broad categories.

First, the concentration of FABP correlates with overall rates of cellular fatty acid uptake, transport, and utilization in several circumstances. In the intestinal epithelium, the concentration of a similar but nonidentical FABP (2) is greater in mucosa from villi than in crypts, in jejunum than ileum, and in mucosa from animals fed a high fat diet than those fed a low fat diet. In liver, the influence of sex steroids on cytosolic triglyceride biosynthesis in rat hepatocytes in suspension increases both hepatic FABP concentration and fatty acid concentrations of a similar but nonidentical FABP concentration and fatty acid uptake (3, 4); also, binding activity of hepatic FABP reportedly is increased in the Zucker obese rat (38), in which total free fatty acid flux is increased. FABP is also present in a wide variety of tissues such as myocardium, adipose tissue, skeletal muscle, and kidney which utilize free fatty acids (1). Finally, we recently have found that a M, 12,000 FABP increases dramatically in the cytosol of 3T3-L1 mouse embryo fibroblasts as they differentiate into adipocytes.

The second line of evidence suggesting a relationship of FABP to cellular fatty acid utilization consists of studies in several laboratories demonstrating an influence of FABP in vitro on the activity of a number of enzyme reactions involved in fatty acid metabolism. Thus, FABP has been shown to enhance the activity of hepatic mitochondrial and microsomal acyl-CoA synthetase (14), peroxisomal fatty acid oxidation (41), microsomal glycerophosphate acyltransferase (14, 42) and diglyceride acyltransferase (43), and acetyl-CoA carboxylase (35). In the intestine, enhancement of microsomal acyl-CoA synthetase (2) and diglyceride acyltransferase (43) has been demonstrated. In several of these studies, the FABP effect was concentration dependent and suggested that variations in FABP concentration within the physiologically relevant range in vivo could modulate enzyme activity.

It should be noted that other evidence is consistent with the possible relationship of these proteins to the metabolism of cholesterol (10, 11), heme (9, 10), and other ligands (9, 31), but in general this evidence is less extensively developed. Even if it is accepted, however, that this closely related or identical group of proteins (FABP, sterol carrier protein, A, Z) is principally related to cellular fatty acid utilization, the nature of that relationship remains unclear. As noted, in vitro evidence is consistent with an FABP effect solely on the interaction of fatty acid substrate with particulate enzymes. In this model, FABP could be viewed either as a vehicle for the efficient transfer of fatty acid to the active site of the enzyme or as a relatively nonspecific "solubilizer." The generally similar effects of albumin in this regard (14) would tend to support the latter interpretation, but the former is by no means excluded. It is also possible that FABP facilitates desorption of fatty acid from the cytosolic aspect of the plasma membrane during uptake, a concept suggested by evidence that desorption may be rate limiting in transport of amphipathic ions such as fatty acids (44-46) and for which preliminary direct experimental support is already available (47). Or, formation of the fatty acid-FABP complex could facilitate the movement of fatty acid through the cytosol, achieving a greater overall net fatty acid flux rate despite a reduced diffusion coefficient by virtue of a greatly increased concentration (14). In regard to these postulated physical effects on the movement of fatty acid within the cell, it may be of particular significance that the abundance of FABP mass in cytosol suggests that expression of its function requires a relatively high molar concentration (estimated from the present data to be 0.4 mM) compared to that of most other cytosolic proteins and that it is involved in processes characterized by high flux rates of relative nonpolar molecules. The flux of long chain fatty acids through liver dwarfs that of other amphipathic substances, as discussed previously (14), and supports the concept that FABP is primarily related to the metabolism of fatty acids rather than that of less abundant moieties such as bilirubin, bile acids, or xenobiotics.

The foregoing considerations notwithstanding, much remains unknown about this protein. Although its cytosolic concentration can be modulated, apparently reflecting changes in synthesis (48), the nature of the effective "signal" for these responses is not defined. Thus, on the one hand, it is equally possible that FABP concentration controls cellular fatty acid flux or, on the other hand, that it is responsive to fatty acid flux. Furthermore, the physical and structural properties of the protein which are essential in its function remain to be defined, as does its possible significance in states of abnormal fatty acid metabolism. These and other fundamental questions about this abundant but still poorly understood protein species are under investigation and only now may be on the threshold of being answered.

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