Characterization of a Fatty Acid-binding Protein from Rat Heart* (Received for publication, December 17, 1985)

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A fatty acid-binding protein has been isolated from rat heart and purified by gel filtration chromatography on Sephadex G-75 and anion-exchange chromatography on DE52. The circular dichroic spectrum of this protein was not affected by protein concentration, suggesting that it does not aggregate into multimers. Computer analyses of the circular dichroic spectrum predicted that rat heart fatty acid-binding protein contains approximately 22% α-helix, 45% β-form and 33% unordered structure. Immunological studies showed that the fatty acid-binding proteins from rat heart and rat liver are immunologically unrelated. The amino acid composition and partial amino acid sequence of the heart protein indicated that it is structurally related to, but distinct from, other fatty acid-binding proteins from liver, intestine, and 3T3 adipocytes. Using a binding assay which measures the transfer of fatty acids between donor liposomes and protein (Brecher, P., Saouaf, R., Sugarman, J. M., Eisenberg, D., and LaRosa, K. (1984) J. Biol. Chem. 259, 13395–13401), it was shown that both rat heart and liver fatty acid-binding proteins bind 2 mol of oleic acid or palmitic acid/mol of protein. The structural and functional relationship of rat heart fatty acid-binding protein to fatty acid-binding proteins from other tissues is discussed.

Fatty acid-binding proteins (FABPs') are a class of small but relatively abundant proteins that are generally thought to be involved in the intracellular transport of fatty acids and possibly other organic anions (1). FABPs from liver (2–4), intestine (5), and adipose tissue (6) have been extensively characterized; the primary structure of each has been determined, either by direct protein sequencing or deduced from the cDNA sequence (3, 7–11). These sequence data have provided a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

The present investigation describes the isolation, characterization, partial amino acid sequence, and fatty acid binding properties of FABP from rat heart.

EXPERIMENTAL PROCEDURES

RESULTS

A description of the isolation, spectral properties, isoelectric point, and amino acid sequences of tryptic peptides of rat heart FABP is presented in the Miniprint Supplement under "Results." The amino acid compositions of rat heart and rat liver FABP determined in the present work are compared in Table I to the amino acid compositions (from cDNA sequences) of FABP from rat liver, rat intestine, and 3T3 adipocytes. All four proteins contain between 122 and 131 residues and are

* Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 4–8, and Table II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-3484, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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enriched with respect to Asx (aspartic acid + asparagine), Glx (glutamic acid + glutamine), and lysine. Rat heart and intestine FABPs lack cysteine, rat liver FABP lacks tryptophan, and 3T3 adipocyte FABP lacks histidine. Rat heart FABP contains significantly more threonine and tryptophan than FABPs from the other three sources.

The circular dichroic (CD) spectrum of rat heart FABP was determined at protein concentrations ranging from 0.22 to 1.8 mg/ml (Fig. 1). The molar ellipticity (θ) at all wavelengths examined was unaffected by protein concentration. The CD spectrum has a large negative ellipticity peak at 216 nm, characteristic of proteins containing a high degree of β-form. An estimate of the content of α-helix in a protein can be obtained from the expression, \( \theta_{222} = [\alpha](\% \text{ helix}) \times -2340 \), where \( [\alpha] \) is the per cent α-helix (24). The molar ellipticity (θ) at 222 nm of rat heart FABP is −9044, from which the calculated content of α-helix is 22.1%.

Computer analyses (25) of the CD spectra of FABP and four reference proteins reveal that the predicted content of α-helix, β-form, and random form in the reference proteins myoglobin, adenylate kinase, papain, and ribonuclease A, are in reasonable agreement with the secondary structure of these proteins based on x-ray diffraction studies (26). Analysis of the CD spectrum of rat heart FABP predicted that the protein contains 22.0% α-helix, 45.0% β-form, and 33.0% random form. These results indicate that heart FABP contains a significant amount of ordered structure.

Fig. 2A shows Coomassie Brilliant Blue-stained gels of rat heart high-speed supernatant, FABP containing fractions from Sephadex G-75, purified heart FABP, and purified rat liver FABP. Fig. 2B shows the autoradiograms of immunoblots of identical gels after electrophoretic transfer onto nitrocellulose paper followed by incubation with rabbit anti-rat heart FABP immune serum and \(^{125}\)I-labeled protein A. Anti-serum directed against rat heart FABP reacted only with heart FABP in the high-speed supernatant, in Sephadex G-75 eluates, and with purified rat heart FABP, but not with rat liver FABP.

In the reciprocal immunoblotting experiment to that shown in Fig. 2, it was found that immune serum directed against rat liver FABP cross-reacts only with rat liver FABP in liver high-speed supernatant, in Sephadex G-75 eluates, and with purified liver FABP, but not with rat heart FABP (data not given). These experiments demonstrate that rat heart and liver FABP are not immunologically related.

The ability of rat heart FABP to bind fatty acids was compared to that of rat liver FABP using a binding assay where each protein was incubated with multilamellar egg lecithin liposomes containing either \([1-\text{14}C]\)oleic acid or \([1-\text{14}C]\)palmitic acid (Fig. 3). It was found that both proteins

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**TABLE 1**

Amino acid composition of FABP from rat heart, liver and intestine, and 3T3 adipocytes

| Amino acid | Rat heart* | Rat liver* | Rat intestine* | 3T3 adipocytes* |
|------------|------------|------------|---------------|----------------|
| Asx        | 14.8       | 11.7       | 20.6          | 19.7           |
| Thr        | 17.8       | 12.1       | 10.0          | 10.1           |
| Ser        | 8.6        | 6.4        | 0.7           | 1.4            |
| Glx        | 12.0       | 17.7       | 15.7          | 16.7           |
| Pro        | 1.3        | 2.5        | 0             | 0              |
| Gly        | 11.0       | 12.6       | 13.4          | 12.7           |
| Ala        | 6.7        | 3.0        | 7.1           | 6.6            |
| Val        | 10.0       | 9.4        | 9.1           | 11.5           |
| Cys        | 0          | 0.6        | ND            | 1              |
| Met        | 2.3        | 6.6        | 0.6           | 4              |
| Ile        | 4.3        | 6.9        | 7.1           | 8              |
| Leu        | 10.0       | 6.8        | 9.0           | 10             |
| Tyr        | 2.2        | 3.2        | 3.2           | 5              |
| Phe        | 6.3        | 6.6        | 8.4           | 8              |
| Lys        | 12.5       | 15.8       | 15.3          | 14             |
| His        | 3.5        | 2.0        | 1.3           | 1              |
| Arg        | 5.2        | 2.3        | 6.0           | 6              |
| Trp        | 2.0        | 0          | ND            | 1              |
| Sum        | 130.5      | 125.9      | 126           | 131            |

* Data from this study based on an average of five or more 24-h hydrolysates assuming an Mₐ of 14,200.
* Data from Ref. 10.
* Data from Ref. 7.
* ND, not determined.

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**FIG. 1. CD spectrum of rat heart FABP.** Spectra were recorded at protein concentration of 1.8 mg/ml (O), 0.9 mg/ml (C), 0.45 mg/ml (C), and 0.22 mg/ml (A) in 5 mM phosphate buffer, pH 7.2. The mean residue ellipticity (θ) from 240 to 202 nm did not change as a function of protein concentration, and the symbols representing θ at selected wavelengths are given to illustrate this result.
been recognized that fatty acid-binding proteins occur in proteins all have similar family on the basis of conditions) to protein was also observed at protein concentration...6 in Miniprint). Both methods consistently yielded approximately 4-8 mg of purified heart FABP from 50 frozen rat hearts. Neither Method 1 nor Method 2 requires a delipidation step, as was used by Fournier and co-workers (16-19), thereby avoiding possible alterations in protein structure produced by exposure to organic solvents. In addition, cation-exchange chromatography, as used by Said and Schultz (20), was not required, thereby avoiding prolonged exposure to acidic pH (less than 6.0), which has been shown to cause instability and irreversible precipitation of rat liver FABP (2).

Although rat heart FABP is related to FABPs from other tissues, it was shown to be a distinct protein by several criteria. The amino acid composition of rat heart FABP, reported here for the first time, shows that this protein is enriched with respect to Asx, Glx, and lysine (Table I) like other FABPs, but is different in that it contains 2 residues of tryptophan, lacks cysteine, and contains significantly more threonine than FABPs from other tissues. Although the obtained amino acid sequences of rat heart FABP tryptic peptides represent only a portion of the primary structure (40 of 131 residues), alignment of these sequences (with gaps to maximize homology) with the amino acid sequence of rat liver FABP showed that partial homology exists between the two proteins, even though their primary structures are not identical. This is not surprising in view of the fact that rat liver and rat intestine FABP are members of the same family of proteins, even though only 34 of 127 amino acid residues are the same (10). Immunoblotting experiments (Fig. 2) revealed that immune serum directed against rat heart FABP does not cross-react with rat liver FABP. This is in agreement with the results of Said and Schultz (20), who showed that immune serum directed against rat liver FABP does not cross-react with rat heart FABP.

Perhaps the most important difference between our findings and those reported by others is related to the aggregation behavior, or lack thereof, of FABPs. Fournier and co-workers (17-19) postulated that porcine and rat heart FABP aggregate into at least four distinct molecular species based on CD and fatty acid-binding isotherms derived from electron spin resonance measurements. We found no evidence for aggregation of rat heart FABP by CD under conditions comparable to those of Fournier and co-workers (17, 18), in that the molar ellipticity (θ) did not vary as a function of protein concentration from 0.22 to 1.8 mg/ml (Fig. 1). It is possible that the delipidation and lyophilization steps used during purification of porcine and rat heart FABP (16-19) may have altered the properties of those proteins, resulting in aggregation which may not occur under physiological conditions.

In our studies, rat heart and rat liver FABP bound both oleic or palmitic acid when these fatty acids were incorporated into liposomes and equilibrated with these proteins (Fig. 3). By incorporating fatty acids into artificial membranes we were able to use relatively high concentrations of fatty acid, which would normally be in the form of an acid soap if added directly to the aqueous phase (32). Perhaps more importantly, the fatty acid-phospholipid complex may provide a somewhat more physiological orientation for the fatty acid, comparable to that in a cellular membrane, as opposed to an aqueous dispersion of a fatty acid ligand. Using our binding assay no major distinctions between oleic and palmitic binding were found. These observations are different from those described previously (20), which indicated that rat liver FABP binds both oleic and palmitic acid, whereas rat heart FABP binds oleic acid but not palmitic acid unless delipidated with butanol. Since conditions used for direct measurement of fatty acid binding in this work differed greatly from those reported elsewhere, comparisons between results are difficult to make. It seems likely that under physiological conditions, both pal-

**FIG. 3. Binding of radiolabeled oleic or palmitic acid to rat heart and rat liver FABP.** Varying amounts of liposomes were incubated with FABP (10 μM) for 1 h at 25 °C, and the fatty acid bound to protein was determined as described under "Experimental Procedures."
mitate and oleate would interact similarly with heart FABP if such interactions were required for normal fuel metabolism.

Reers et al. [21] used fluorescence to monitor the movement of (O'-antrolxoyl)palmitic acid between vesicles and bovine liver and heart FABP. It was found that bovine heart FABP will only donate the ligand to vesicles, whereas bovine liver FABP removes the ligand from vesicles, suggesting different affinities of the respective proteins for C16 fatty acids. Again, the conditions used in these studies differed considerably from ours, in which radiolabeled fatty acids rather than fluorescent analogues were used, and in which movement of fatty acids from synthetic bilayers to both rat heart and rat liver FABP was demonstrated.

Several previous studies, including one from this laboratory (22, 33, 34), have reported that FABPs bind an equimolar amount of fatty acid in experiments where the protein concentration was determined by either the Lowry et al. (35) or Bradford (36) procedure. In the present work the concentration of FABP in the binding assays was determined by quantitative amino acid analysis, and it was consistently found that both rat heart and rat liver FABP bind 1.8-2.0 mol of fatty acid/mol of protein (Fig. 3). It is recognized that quantitative amino acid analysis is the best way to determine accurately the concentration of a protein in solution. It is also well known that spectrophotometric (A_{280}) and colorimetric assays using bovine serum albumin as the standard can frequently underestimate or overestimate the concentration of a particular protein (37). Therefore, when it is desirable to determine binding stoichiometries between proteins and ligands, caution should be exercised when protein concentrations are determined by spectrophotometric or colorimetric methods.

It is noteworthy that our binding assay measures the partitioning of a fatty acid between donor liposomes and acceptor protein after equilibrium has been reached, and that protein-bound and free (unbound) fatty acids are separated by gel filtration (34) or using Lipidex (22, 39). These procedures may result in removal of weakly bound fatty acids, thereby lowering the apparent number of fatty acids bound to the protein. Consequently, the method selected for measurement of protein concentration and the procedure used for measuring fatty acid binding may explain why we observe that rat heart and rat liver FABPs bind approximately 1 mol of fatty acid/mol of protein, whereas earlier studies (22, 33, 34) have indicated that FABPs bind only 1 mol of fatty acid.

Finally, examination of the primary structure of rat and human liver FABPs has shown that these proteins are comprised of two homologous half-molecules, each of which contains two tandemly repeated sequences (8). This suggests the possibility that both half-molecules may contain a ligand binding domain. The latter observations may provide a structural basis for our finding that rat heart and rat liver FABPs bind 2 mol of fatty acid/mol of protein.

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In this work, circular dichroic spectra of purified rat heart and liver fatty acid binding protein (FABP) were recorded at protein concentrations of 3-10 μM. Spectra of purified rat heart and liver FABP were obtained using a Spex spectrophotometer equipped with a Spex 7500 spectrophotometer. FABP was isolated from homogenized rat hearts and partially purified by gel chromatography on Sephadex G-75. Overall, the yield of the partially purified FABP was 30-50% of the initial amount.

The circular dichroic spectra of rat heart FABP were determined at 25°C using a Cary 61 spectrophotometer. Circular dichroic spectra were recorded in a 0.1-cm pathlength cuvette containing a 10 μM solution of FABP in 50 mM Tris-HCl, pH 7.4, and rechromatographed on DE-52 under the same conditions. FABP was isolated from homogenized rat hearts and partially purified by gel chromatography on Sephadex G-75. Overall, the yield of the partially purified FABP was 30-50% of the initial amount.

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Inositol trisphosphate (InsP$_3$) is known to evoke the release of Ca$^{2+}$ from the endoplasmic reticulum (ER) in various cells. However, the mechanism of InsP$_3$-induced Ca$^{2+}$ release remains unclear. We investigated the role of InsP$_3$-mediated Ca$^{2+}$ release in rat hepatocytes using a fluorescent indicator, indo-1. The results indicated that InsP$_3$ induces a transient increase in intracellular Ca$^{2+}$ concentration, supporting the idea that InsP$_3$ might be a key regulator of Ca$^{2+}$ signaling in hepatocytes.

**Proposed Mechanism:**

1. InsP$_3$ binds to its receptor on the ER membrane, activating the receptor.
2. The activated receptor opens ion channels, allowing Ca$^{2+}$ to flow into the cell from the extracellular space.
3. The increase in intracellular Ca$^{2+}$ concentration triggers a series of biochemical reactions, including the activation of various enzymes and the translocation of proteins.

**Implications:**

Understanding the role of InsP$_3$ in Ca$^{2+}$ signaling is crucial for elucidating the physiological functions of hepatocytes and for developing strategies to modulate Ca$^{2+}$ signaling in liver diseases.