Cellular Fatty Acid-Binding Proteins

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Cellular Fatty Acid-binding Proteins

edited by

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Preface

Fatty acid-binding proteins (FABPs), small proteins abundantly present in cells of mammalian tissues, were discovered in 1972. During the past fifteen years these proteins have been the subject of research in an increasing number of laboratories. Although their precise physiological role has not yet been elucidated in great detail, the information available allows the conclusion that FABPs play an essential role in the regulation of lipid metabolism and transport in liver, kidney, heart, skeletal muscle and intestine.

Progress in this field requires close communication among workers of various disciplines. The overwhelming amount of new information that has recently become available has prompted us to organize, for the first time, a workshop especially dedicated to this fascinating protein. The main objectives of the workshop were to provide biochemists, biophysicists, physiologists and clinicians a review of 'the state of the art' of FABPs and to present and discuss their latest research findings. This volume is based on selected papers from the conference. It includes contributions of many of the leading investigators and presents the most recent developments in this active area of research. It is our sincere hope that these articles will form a stimulus to future research on fatty acid-binding proteins so as to bring us to a better understanding of these fascinating proteins and their significance for the functioning of the cell.

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Historic overview of studies on fatty acid-binding proteins

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Key words: fatty acids, fatty acid-binding protein, peroxisomes, β-oxidation, intestine, liver

Summary

Fatty acid-binding proteins (FABPs) were first identified in the cytosol of rat intestinal mucosa during studies on the regulation of intestinal fatty acid uptake. The subsequent finding of FABP activity in the cytosol of many other tissues initially was believed to reflect a single protein. However, the FABPs are now recognized as products of an ancient gene family comprised of at least 9 structurally related, soluble intracellular members, a number of which exhibit high-affinity binding of long-chain fatty acids. Despite recent insights into regulation and tissue-specific expression suggesting FABPs to subserve diverse roles, their precise biological functions remain to be elucidated.

Introduction

It is gratifying that the fatty acid-binding proteins (FABPs) have emerged as a topic of sufficient importance to warrant an international conference, and especially one attended by so many outstanding contributors to this rapidly evolving field. Because of their efforts, I believe that we are now at the threshold of an understanding of the real significance of these small but abundant and closely regulated intracellular proteins.

In this paper I will give a short historical summary, consider some of the current questions and possibilities concerning FABP function and regulation and, in this context, briefly describe some of the questions we are currently addressing in our own laboratory.

Discovery of fatty acid-binding proteins

The first experiments concerned with FABP were conducted in 1971 [1]. They were designed to test the hypothesis that certain unexpected observations made in studies of the intestinal absorption of long-chain fatty acids might be accounted for by a soluble fatty acid carrier in the enterocyte. Those experiments, employing Sephadex chromatography of intestinal mucosal cytosol, indeed demonstrated noncovalent binding of [^{14}C]oleate to a low-molecular-weight cytosolic protein, the distribution of which we later showed corresponded precisely to that expected for a relationship to fat absorption.

In the belief that a cytosolic protein of this kind could have very broad implications for cellular lipid metabolism, we surveyed other tissues known to utilize fatty acids. Binding activity proved to be even more prominent in liver cytosol than in intestine, and was demonstrated in other tissues as well. At this point, before FABP had been purified, the simplest and most attractive interpretation was that a single protein accounted for cytosolic fatty acid binding in all tissues.

Unfortunately, this interpretation was too simple. Our subsequent isolation of what is now designated intestinal fatty acid-binding protein or I-FABP, and the preparation of a specific antibody to it [2], made it clear that the intestinal protein was absent from liver cytosol, and implied that the

abundant binding activity in liver must be accounted for by a different protein. Incontrovertible evidence for this interpretation subsequently evolved from a productive collaboration that developed in 1981 between our laboratory and that of David Alpers and Jeffrey Gordon at Washington University in St. Louis. I believe that this collaboration properly can be considered a watershed in the history of FABP research, and the dawn of its molecular era.

In their studies of enterocyte protein synthesis in a cell-free system, Gordon, Alpers, and Strauss had found that the most abundantly expressed messages were for two then unidentified low-molecular-weight proteins. Alpers contacted me, wondering if one of them might be FABP. Since we had very recently isolated and characterized liver FABP and prepared an antibody to it [3], we were able to provide the St. Louis group with both liver and intestinal FABP and their respective antibodies.

The abundant expression of both forms of FABP in intestine, and the absence of the intestinal form from liver, was quickly established [4, 5]. The St. Louis team and later others went on to characterize the amino acid and nucleotide sequences of these and other related proteins, and recognized important homogeneities among them and with the cytosolic retinoid-binding proteins.

FABP as member of a protein family

The broader gene family now numbers 8 or 9 members which differ in structure and organ distribution (Table 1). Much of the homogeneity appears to reside in the first exon of a closely conserved overall gene structure consisting of 4 exons and 3 introns, in addition to the flanking regions. The acetylated N-terminus and the absence of a signal peptide sequence are typical of proteins destined for an intracellular location.

Chan [6] has estimated that the 'modern' FABPs initially diverged from a common progenitor around 1 billion years ago, i.e. prior to the emergence of the vertebrates, while I-FABP diverged from L-FABP about 600 million years ago, before the mammals evolved. The recent studies of Sacchettini [7], Cistola [8], Storch [9], and their colleagues have clarified much of the nature of the binding of fatty acids by FABP. This binding is clearly not an incidental property, but rather one for which the proteins seem to have specifically evolved.

In fact, it is not at all surprising that such proteins should have evolved, given the lengths to which nature seems to have gone to assure that wherever long-chain fatty acids exist, they are largely complexed to proteins, whether extracellular, in transit through the plasma membrane, or intracellular. In this respect the recent discoveries of the plasma membrane fatty acid-binding protein [10, 11], and the acyl-CoA binding protein [12] should be noted.

Biological role of FABP

What then is the particular mission of the FABPs? Are they only to bind and transport fatty acids? As they diffuse down one physicochemical gradient for the complexed form and in the reverse direction for the uncomplexed form, are FABPs simply passive vehicles for the poorly soluble fatty acid molecules? This was a major tenet of our initial hypothesis, yet such a view would hardly seem to justify the evolution of so broad and so complex a gene family, and certainly would not account for the presence of two distinct and independently regulated FABPs in the same cell, as occurs in intestine [13].

Table 1. FABP gene family

Protein	Tissue expression
1. L-FABP (hFABP, Z, A, Band C, SCP)	liver, intestine
2. I-FABP (gFABP)	intestine
3. CRBP	widespread
4. CRBP II	intestine
5. CRABP	widespread
6. (CRABP II)	(uncertain)
7. p422 (aP2)	adipose tissue
8. Myelin P2 protein	central, peripheral nerve
9. M-FABP (H-FABP, cFABP)	heart, brain, skeletal muscle, widespread

Rather, while acknowledging that we still do not understand the function of any of the FABPs with certainty, it seems necessary to consider a broader array of possibilities (Table 2):

1. Binding of fatty acids, by definition, may be the principal function of FABP. All other functions can be considered in a sense derivative.

2. Fatty acid transport, either within the cytosol or between membranes and cytosol, remains attractive, as it was at the outset. This function has been neither established nor excluded, although the work of Storch and Kleinfeld [14] suggests that for fatty acids moving through membranes it is flip-flop rather than desorption that is rate-limiting.

3. There is abundant evidence from many laboratories that FABP may serve as a cofactor, enhancing the rates of various synthetic or oxidative reactions that are fundamental to normal fatty acid metabolism. This function may be subserved either by FABP monomer or possibly a dimer or an oligomer [15]. While it is difficult in some cases to determine to what extent these various observations reflect events in the intact cell and whole animal, I believe that most of us regard these observations as very relevant indeed.

4. Compartmentalization of fatty acids certainly occurs, at least, in the enterocyte, as was shown by the experiments conducted some time ago by Gangl and co-investigators [16]. In these studies, [^{3}H]-palmitic acid was administered intra-luminally, simultaneously with the intravenous administration of the [^{14}C]-labeled compound. As expected, the intraluminal fatty acid was predominantly incorporated into triglyceride; fatty acids derived from plasma, however, were preferentially oxidized or incorporated into phospholipid. Could this striking intracellular metabolic compartmentalization reflect the presence and differential targeting of two abundant FABP species? This fascinating and highly significant issue remains unresolved.

Table 2. Putative functions of FABP

1. Bind fatty acids
2. Transport fatty acids (in cytosol, or between membrane and cytosol)
3. Serve as cofactor for reactions in which fatty acid or acyl-CoA are substrates
4. Affect intracellular compartmentalization of fatty acids
5. Influence mitosis, cell growth
6. Participate in insulin signal transduction
7. Contribute to heme, selenium metabolism, sterol biosynthesis
8. Protect against adverse effects of long chain fatty acids, acyl-CoA

5. Very exciting new observations, independently emanating from the laboratories of Sorof in Philadelphia [17] and Grosse [18] in Berlin suggest a relationship of liver and muscle FABP, respectively, to mitosis and cell growth in liver and in breast cancer-cell culture (cf. 19). Their pursuit promises to illuminate much that is presently unknown about the FABPs, and very likely the processes governing cell replication and growth in general.

6. Other fascinating studies, performed by Lane and colleagues at Johns Hopkins have identified the FABP-related aP2 (or 422) protein of 3T3-L1 adipocytes as a phosphorylated intermediate that plays an essential role in insulin signal transduction [20]. In these experiments the phosphorylated protein, designated as pp15 (for 15 kDa phosphorylated protein), was allowed to accumulate by inhibiting its dephosphorylation with phenylarsineoxide, thereby also blocking the stimulatory effect of insulin on the glucose transporter in these cells. One is tempted to speculate that the phosphorylation and dephosphorylation of this particular FABP also influence its fatty acid-binding affinity, perhaps in a way that coordinately contributes to the regulation of both glucose and fatty acid flux in response to a continually changing balance between insulin and glucagon dominance. This interesting possibility also remains unexplored.

7. Liver FABP may also play a role in heme and selenium metabolism and sterol biosynthesis. Little is known about these interactions, and a relationship between them and fatty acids at the level of FABP is not apparent at this time.

8. Special consideration should be given to the possibility that the FABPs serve to protect the cell against undesired and potentially catastrophic effects of the long-chain fatty acids themselves. It

would not be difficult to design an experiment showing that fatty acids disrupt almost any biological function imaginable. At high concentrations these surface-active compounds could easily alter membrane function or the activity of any process in which lipid-protein interactions play a role. Thus, much of this literature must be viewed with skepticism, justified by the concern that in vitro experiments may not realistically reflect conditions in the intact cell. Nonetheless, a number of studies involving fatty acid concentrations in the range of 10^{-5} to 10^{-6} M, provide convincing evidence that, under physiological conditions, vital processes may in fact be susceptible to modulation or even disruption by fatty acids. Potentially vulnerable processes include control of ion fluxes, enzymes involved in cell regulation, energy production (including the physiologically useful thermogenesis in brown adipose tissue), and even processes important in the regulation of gene expression. A few examples will serve to illustrate this point.

In studies of amino acid transport in rat brain synaptosomes, conducted with our colleagues in neurosciences in San Francisco [21], exogenous fatty acid was excluded from the system. The experiments showed that the presence of FABP was associated with an increase in sodium-coupled amino acid transport, related to removal of endogenous membrane fatty acids. There was no effect on sodium-independent amino acid transport. Thus, in this example, even the modest amount of endogenous fatty acid already present in the membrane was sufficient to influence a coupled-transport process.

In a different area, van der Klis *et al.* [22] have recently shown that nuclear binding affinity for T3 is significantly diminished by oleic acid at concentrations between 1 and 10 μM, implying that fatty acids could modulate or perturb thyroid hormone regulation of gene expression.

Finally, Goodfriend and Ball [23] have shown that the interaction of angiotensin II with its receptor in bovine adrenal glomerulosa cells is significantly affected by oleic acid or arachidonic acid, even at concentrations as low as 3 μM. Although this is a surface membrane process, and thus effectively extracellular, it underscores the concept that fatty acids may be appropriately viewed as biological two-edged swords. Perhaps in analogy to Na^+, Ca^{++}, and oxygen itself, they are essential for normal cellular function, but they must be closely regulated and compartmentalized. Although evidence against this concept has been reported by Glatz *et al.* in studies of ischemic myocardium [24], it remains a possibility at least in other tissues.

Despite these and other clues to many possible functions of the FABP, their relevance to specific physiological or pathophysiological phenomena is often obscure. Moreover, it is likely that the functions of the FABP differ not only among the proteins themselves and the tissues in which they reside, but also under the influence of changing metabolic conditions, the cell cycle, and other potential regulatory factors, both endogenous and exogenous.

Regulation of FABP gene expression

Some particularly useful insights into FABP function have been provided by the substantial progress that has been made in FABP regulation. In any consideration of this area, of course, we cannot fail to recognize the important recent advances by Sweetser *et al.* [25, 26], Hunt *et al.* [27], and others, in elucidating the location and function of FABP gene regulatory sequences (cf. 28).

In many instances in which FABP expression is regulated, a relationship to changes in cellular fatty acid flux is very evident, while in others it is less obvious or inapparent. It is significant that in the regulation of intestinal FABP by dietary fat intake [2], the change in fatty acid flux is primary, while the response in FABP expression is secondary. In essentially all instances thus far studied, changes in FABP abundance, in turn, reflect parallel changes in FABP mRNA abundance. This evidence and studies of labeled leucine and [^{14}C]-carbonate incorporation indicate that FABP regulation largely reflects changes in synthesis rather than in turnover. Although this pattern of responsiveness of FABP production to changing fatty acid flux could be regarded as consistent with a mechanism which protects the cell against adverse fatty acid effects, it

certainly neither constitutes proof, nor excludes other functional advantages.

A particularly significant aspect of L-FABP regulation is that related to the effects of clofibrate [13], the response to which is limited to L-FABP in liver and intestine; for practical purposes, it is not shared by I-FABP. Thus, these demonstrate differential regulation of I-FABP and L-FABP in the enterocyte, implying that these proteins subserve different functions. This interpretation is particularly interesting in view of the compartmentalization of luminal and plasma-derived fatty acids in the enterocyte, but, as noted earlier, a connection between the two has not been established.

FABP and peroxisomal fatty acid oxidation

Kawashima and colleagues [29] earlier had shown a striking correlation between the extent to which L-FABP and peroxisomal β-oxidation in liver were induced by fibrates of varying potency. This parallel regulation between L-FABP and the peroxisomes suggests that a functional relationship exists between them.

Unlike that in mitochondria, β-oxidation in peroxisomes is not dependent on carnitine for its initiation, and the resulting electron transfer leads to the formation of hydrogen peroxide. After several β-oxidation cycles, during each of which an acetyl-CoA is generated, the chain-shortening ceases with the formation of octanoyl-CoA. Both acetyl-CoA and octanoyl-CoA are converted to their respective acyl-carnitines and undergo further oxidation in the mitochondria. The first suggestion that FABP might participate in this process was based on early, somewhat indirect evidence provided by Appelkvist and Dallner [30], and is an area in which we are currently interested.

The fibrate drugs also induce the activity of the cytochrome P452-mediated fatty acid ω-oxidation system in the endoplasmic reticulum [31]. The ω-hydroxy fatty acids thus produced are converted by an additional oxidative step in cytosol to their dicarboxylic acid analogs, which in turn are oxidized preferentially in the peroxisomes. This bipartite extramitochondrial oxidizing system thus is induced by fibrate drugs and other peroxisome proliferators at the transcriptional level, and also exhibits increased activity under conditions of fatty acid excess. Unlike the stringently controlled rate of β-oxidation in mitochondria, this system is not subject to direct hormonal regulation. As a result, it could serve as a pathway for the oxidative disposition of fatty acids which might be present in excess of a potentially limited mitochondrial capacity.

In the context of this background, I would like to briefly mention some recent experiments performed in our laboratory by Ruth Brandes, Tony Bass, and more recently Raja Kaikaus, with the technical assistance of Nina Lysenko and Joan Manning [32]. In these experiments, the apparent relationship of peroxisomal β-oxidation to L-FABP was investigated using primary hepatocyte cultures. Fibrate induction of peroxisomal β-oxidation in hepatocyte cultures had already been demonstrated in other laboratories. In contrast, induction of L-FABP in an in vitro system had not been described, yet it could be extremely useful for studying FABP regulation and function. In these experiments, primary hepatocyte cultures were exposed to either bezafibrate or clofibrate, and the activity of peroxisomal (i.e. cyanide-insensitive) palmitoyl-CoA oxidation and the abundance of FABP determined immunochemically were quantified at intervals.

It was found that the time course of the responses to bezafibrate in culture differed greatly. Thus, peroxisomal β-oxidation increased dramatically to near peak values between days 2 and 3, whereas FABP abundance exhibited a more gradual rise, sustained throughout the 9-day duration of the experiment. Authenticity of the FABP response was shown by the increased abundance of FABP relative to controls at 3, 6 and 9 days, both in gels and in a Western blot employing purified L-FABP and a specific L-FABP antibody. As in other settings, enhanced FABP expression in hepatocyte cultures induced by bezafibrate was associated with an increase in FABP mRNA, as shown by Northern blotting using an L-FABP cDNA probe (kindly provided by Dr. J. Gordon). L-FABP mRNA was very abundant at 5 days in bezafibrate-exposed cells, whereas it was undetectable in 5-day control

cells; a blot of a γ-actin cRNA probe was used as a control.

Thus, these studies show that L-FABP expression can be induced in primary hepatocyte cultures by fibrate drugs, reflecting increased mRNA abundance. The time course of FABP induction differs from that of peroxisomal β-oxidation, allowing for the possibility – but not establishing – that their mechanisms of induction differ. Primary hepatocyte culture provides a very useful model for study of in vitro regulation of L-FABP expression in general and its relationship to extramitochondrial oxidative pathways in particular. We are actively employing this system in our ongoing studies.

Concluding remarks

In conclusion, it is clear that research on fatty acid-binding proteins has produced remarkable and continuing advances. Perhaps the progress that we can now anticipate in regard to the function of the FABP will match the very striking recent breakthroughs in the elucidation of its structure, molecular genetics, and regulation.

What can we say at this point about FABP function? The evidence for a correlation between FABP and cellular fatty acid flux and utilization stands, as noted, with few exceptions, and can be related to several transport, esterification, or oxidative processes. In regard to the latter, the implications of the apparent relationship of L-FABP to peroxisomal function seems especially intriguing, as does a primary role for the FABP in protecting vital processes from unacceptably high concentrations of unbound fatty acids. The exciting new evidence for a relationship of FABP to cell growth and to signal transduction will certainly stimulate important new insights in these areas.

The presently available evidence notwithstanding, several questions still remain unanswered. Exploration of these remaining issues in future years will undoubtedly afford an even clearer and more complete understanding of these elusive proteins and their special contributions to the life of the cell.

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Detection, tissue distribution and (sub)cellular localization of fatty acid-binding protein types

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Key words: fatty acid-binding protein, tissue distribution, ligand-FABP interaction, muscle, kidney

Summary

This overview of recent work on FABP types is focussed on their detection and expression in various tissues, their cellular and subcellular distribution and their binding properties. Besides the 3 well-known liver, heart and intestinal types, new types as the adipose tissue, myelin and (rat) renal FABPs have been described. Recent observations suggest the occurrence of more tissue-specific types, e.g. in placenta and adrenals. Heart FABP is widely distributed and present in skeletal muscles, kidney, lung, brain and endothelial cells. The cellular distribution of FABP types appears to be related to the function of the cells in liver, muscle and kidney. The presence of FABP in cellular organelles requires more evidence. The functional significance of the occurrence of more FABP types is unclear, in spite of the observed differences in their ligand-protein interaction.

Abbreviations: FABP(s), Fatty Acid-Binding Protein(s)

Introduction

Our interest into FABPs arose with the study of muscular fatty acid oxidation. Which mechanism would be responsible for the transport of the water-insoluble fatty acid from the plasma membrane to the intracellular organelles where oxidation takes place? On their way from the capillary to the mitochondria or peroxisomes, fatty acids have to pass a series of aqueous spaces and membrane barriers [1]. They have to be transfered to the interstitial space through clefts of the endothelium or through the endothelial cells. Albumin was thought to play a role in this latter process, but FABP may also be involved in the transcellular transfer. Recently we could establish the presence of FABP by ELISA in cultured endothelial cells of man and rat [2], as was earlier observed by Fournier and Rahim [3] in the intact rat heart by immuno-electron microscopy. We could not detect FABP in blood and very little in interstitial fluid.

Several models have been proposed for the cellular mechanism of uptake of fatty acids: passive diffusion, interaction with an albumin receptor or with a plasma membrane fatty acid-binding protein, which is quite different from the cytosolic FABP [1]. This latter molecule may also have an important function in the cellular uptake by increasing the transbilayer movement of fatty acids due to their release from the inner half of the plasma membrane and translocation to FABP [1]. Detection and quantitation of FABPs in cytosols have been performed by a variety of assay methods (Table 1), that each have their specific advantages and shortcomings, not quantitative and aspecific as the coelution and coelectrophoresis methods, not

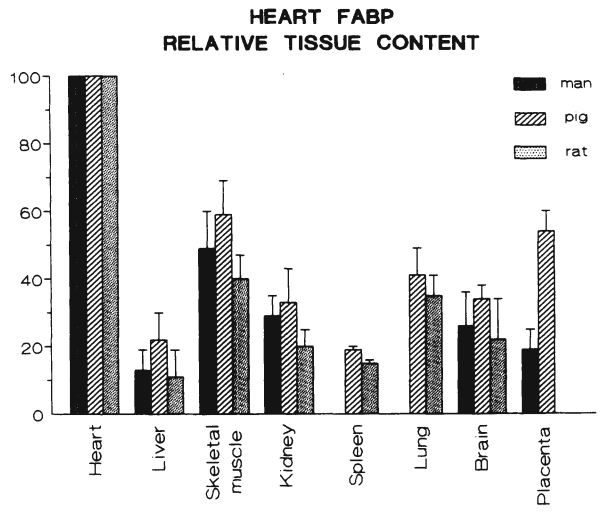

Fig. 1. Relative immunoreactivity with anti-heart FABP antiserum of cytosolic proteins from various tissues of man, pig and rat. Values (determined with antiserum against species-specific heart FABP) are given in % of the reaction of heart cytosol. Data are obtained from ref. [2].

appropriate for determination of binding parameters as affinity chromatography and the immunochemical assays, not discriminative between more types of fatty acid-binding proteins as the radiochemical and fluorescence assay of binding capacity. Fluorescence, electron spin resonance and nuclear magnetic resonance spectroscopy are especially adequate for study of binding parameters of pure FABP preparations.

Detection and tissue distribution of FABP types

The FABP types described up to now belong to a family of low-molecular-weight cytosolic proteins, which show a large extent of structural homology and bind hydrophobic ligands [4, 5].

Most investigations have been directed to the three FABP types, originally described according to the tissue from which they were first isolated, as liver, intestinal or heart FABP. With species-specific antibodies against liver FABP it could be shown, that only liver tissue and intestine of man, pig and rat contain this type of FABP [2, 6, 7]. In an enzyme-linked immuno assay the heart type appears to be present with a comparable relative content in skeletal muscle, kidney, lung, brain and placenta of these 3 species (Fig. 1). Immunoreactivity with anti-heart FABP antiserum was observed in skeletal muscle, kidney, brain, aorta, mammary gland, ovaries, testes and adipose tissue of the rat [6–13]. The presence of heart FABP type in rat kidney has been established by Lam *et al.* [8]. We isolated heart FABP type from human kidney. The presence of rat heart FABP mRNA was demonstrated in rat skeletal muscle, brain, testes, placenta, adrenals and kidney [14] and in aorta [13], but not in spleen and lung [14]. The renal concentration of heart FABP mRNA showed a remarkable, decreasing pattern at postnatal development of the rat, although the FABP concentration remained constant [14]. The intestinal FABP type was only present in the duodenum, jejunum and colon [6].

A comparison of the fatty acid-binding capacity and the immunoreactivity with anti-rat heart and liver FABP antibodies of cytosols from various rat tissues (Fig. 2) demonstrates the inability to determine FABP in many tissues with only anti-liver or anti-heart antibodies. The FABP content of e.g. kidney, adrenals and brain is grossly underestimated, probably due to the presence of other FABP types. The fatty acid-binding activity cannot be in accordance with the FABP content, when additional fatty acid-binding proteins as albumin, α-fetoprotein or others are present, as investigations on postnatal development of FABP in rat heart and liver have illustrated [7]. In our binding assays cytosolic preparations were always liberated from albumin by immuno-affinity chromatography. The con-

Table 1. Assays of fatty acid-binding protein(s)

Coelution on gelfiltration
Electrophoretic comigration
Immunological assay with specific antibodies
Affinity chromatography
Equilibrium dialysis
Binding of fluorescent fatty acid analogue
Transfer of labeled fatty acid from (multilamellar) liposomes
Radiochemical binding assay (charcoal, Lipidex 1000)
Nuclear magnetic resonance
Electron spin resonance

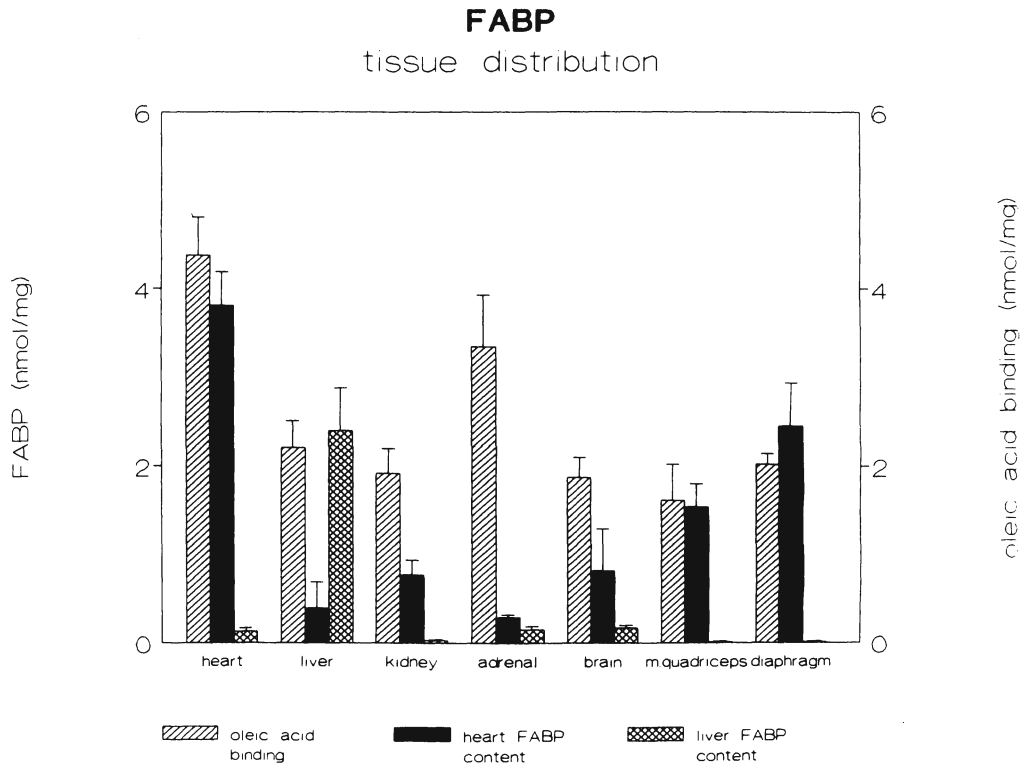

Fig. 2. Comparison of the oleic acid-binding capacity and the immuno-reactivities with anti-rat heart and liver FABP antibodies of cytosolic proteins from various rat tissues. Values are taken from refs. [7] and [34].

tent of α-fetoprotein can, however, not explain the difference between the fatty acid-binding capacity and the immunochemically determined FABP content.

These data indicate the need to apply different assays for the FABP content of tissue cytosols and to look for the presence of (more) different FABP types. A systematic survey of tissues on their FABP composition seems therefore important. FABPs have been isolated from the cytosols of a number of tissues of different species, mostly by a sequence of gelfiltration and ion exchange chromatography [1]. The usual criterion of the purification procedures was to obtain a pure protein. Only in some cases the specific binding activity was followed [15–17]. The isolated FABP preparations from liver have generally a higher binding activity than those from other tissues (Table 2). Pure preparations from some tissues as rat and porcine skeletal muscle and human placenta lacked or had only a very low binding activity. The fatty acid/FABP molar ratio was always less than one. The isolation procedures employed for FABPs from various sources reflect the structural differences of the different FABP

Table 2. Fatty acid-binding activity of FABP preparations from human, porcine and rat tissues

Origin	Man	Pig	Rat
Liver A isoform	51	42	41
B isoform	45	44	34
Heart	26	15	27
Muscle	24	1	10
Kidney (renal type)	24		
(heart type)	19		25
Intestine (liver type)	41		
(intestinal type)	23		
Placenta	1		

Values are given in nmol/mg protein as means for 2–4 preparations. FABP preparations were isolated according to procedures described previously [15–17, 20]. Activity was measured at 1 μM [1-^{14}C]oleic acid by the Lipidex assay, protein was determined by quantitative amino acid analysis.

types. Besides the liver, intestine and heart types, recently three other types were described; kidney [8], adipose tissue [18], and myelin [19]. The primary structure of these proteins (from rat, human or murine origin) is known, except for the kidney type. They show 30–67% amino acid sequence homology with rat heart FABP [1, 5]: especially the amino terminal part of the protein and the first exon of the FABP gene have a marked high degree of homology.

Recent observations on FABP in muscle, kidney and placenta

We will limit us further to some data we obtained on FABP preparations from human skeletal muscle, kidney and placenta. DEAE-cellulose chromatography delivered a human muscle FABP preparation, contaminated with myoglobin, which could be eliminated with CMC-chromatography [16]. Its physico-chemical characteristics (molecular mass, iso-electric point, amino acid composition) and its immunological properties appear similar to heart FABP [16], but definitive evidence on their similarity is lacking.

Human kidney cytosol shows on DEAE-Sepharose chromatography of its low-molecular weight fraction two fatty acid-binding peaks [20] as human liver cytosol, but in contrast to the liver [19] they do not represent isoforms, but different FABP types as was already observed for rat kidney [8]. Both types were present in kidneys from males and females, which is in contrast to observations on a rat kidney FABP identical with α_{2U}-globulin, which was only present in male kidney [21]. After further purification, both types show clear differences in various respects: molecular characteristics, binding properties, immunological reactivity and distribution within the kidney [20]. Peak A looks similar to human liver FABP, peak B to human heart FABP, as for example the fluorescence spectrum with dansylamino-undecanoic acid binding shows a shift of emission and excitation wave lengths and a large increase with the A type, no shift and a slight increase with B. Kidney FABP A binds various ligands like liver FABP. Peak A cross-reacts strongly with anti-liver FABP antiserum, liver FABP with anti-kidney FABP A serum. A corresponding cross-reactivity was observed between kidney FABP B and heart FABP and their antisera.

Human placenta appeared to contain 3 isoforms of a FABP type, which gave only a slight cross-reaction with antisera against the FABPs from liver, heart and kidney. The evidence for the existence of new FABP types in human kidney and placenta and the similarity of human muscle FABP with the heart type await structural analysis of the amino acid sequence or cDNA of these proteins.

Ligand-FABP interaction

There are a large number of differences between FABP types (Table 3). We cannot discuss these in detail. Differences in ligand binding specificity are well-known for liver and heart FABPs [1]. The former molecules appear to bind various types of ligands, among which haem has a high affinity. Other FABPs bind only fatty acids or their CoA- or carnitine esters [15]. The fatty acid binding stoichiometry is variable for liver FABP [1] and dependent on the assay conditions [22], but equals one for intestinal and heart FABP. The binding affinity for C_{16}–C_{18} acids (Kd value 0.2–1 μM) and for fluorescent fatty acids is comparable for all three types [1, 15, 17]. Liver and intestinal FABPs show, however, different interactions with the fatty acid ligand [22]. The binding environment of liver FABP is more solvent-accessible, the bound

Table 3. Differences between FABP types

Amino acid composition and sequence (30–70% homology)
Ligand binding
– specificity
– stoichiometry
– site and interaction
Regulation of synthesis and turnover
Chromosomal location
Tissue expression
Functional properties?
Subcellular distribution?

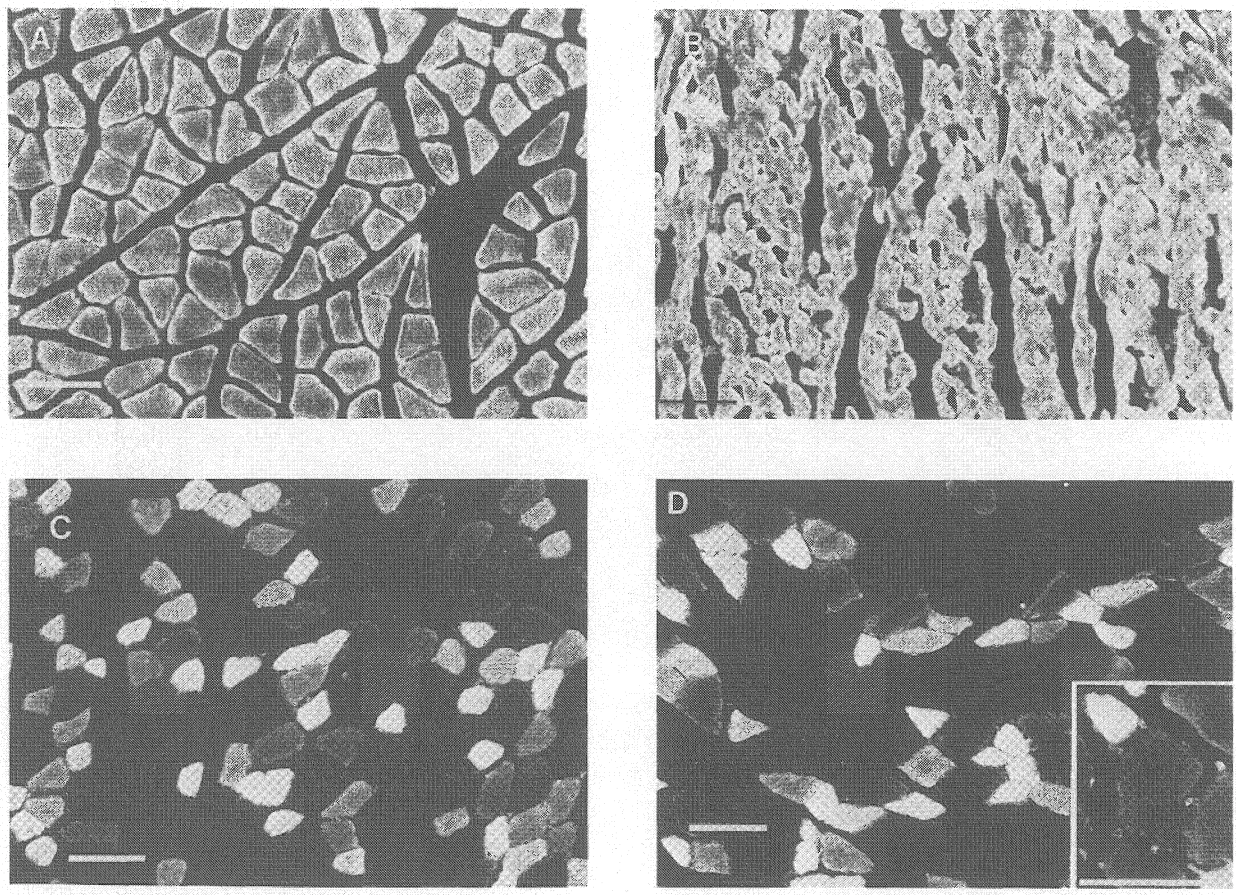

Fig. 3. Immunofluorescent localization of FABP in rat muscles with anti-rat heart FABP antiserum. Muscles from young adult, male Wistar rats were fixed in 2% formaldehyde: m. soleus (a), heart (b), m. extensor digitorum longus (c), m. quadriceps (d). Note that the intensity of staining is inversely proportional to the diameter of the fibers in c and d. Inset: capillary endothelial cells are immunopositive. Bar: 100 μm.

fatty acid shows an ionization shift and its carboxyl group appears to be located at or near the solvent/protein interface, unlike intestinal FABP [22].

Cellular and subcellular distribution

The FABP types are not only limited to certain tissues, but also show a specific cellular distribution in relation with their involvement in fatty acid metabolism. Liver FABP is only present in hepatocytes of foetal and adult human and rat liver [23], and shows a periportal preference in adult liver [24]. Both liver and intestinal FABP types show a gradient in the intestine from duodenum to colon and from crypt to villus tip [25].

In accordance with the observed relation between FABP content and fatty acid oxidation capacity [16] the distribution of FABP in rat skeletal muscles is related to the muscle fiber type. Immunohistochemical observations on rat m. soleus, m. extensor digitorum longus (EDL) and m. quadriceps show that slow oxidative and fast-oxidative-glycolytic fibers react strongly with anti-rat heart FABP antiserum, whereas staining of fast glycolytic fibers is absent or weak (Fig. 3). The intensity of staining correlates with succinate dehydrogenase activity [26]. In m. EDL small fibers react strongly, whereas those with intermediate and large dia-

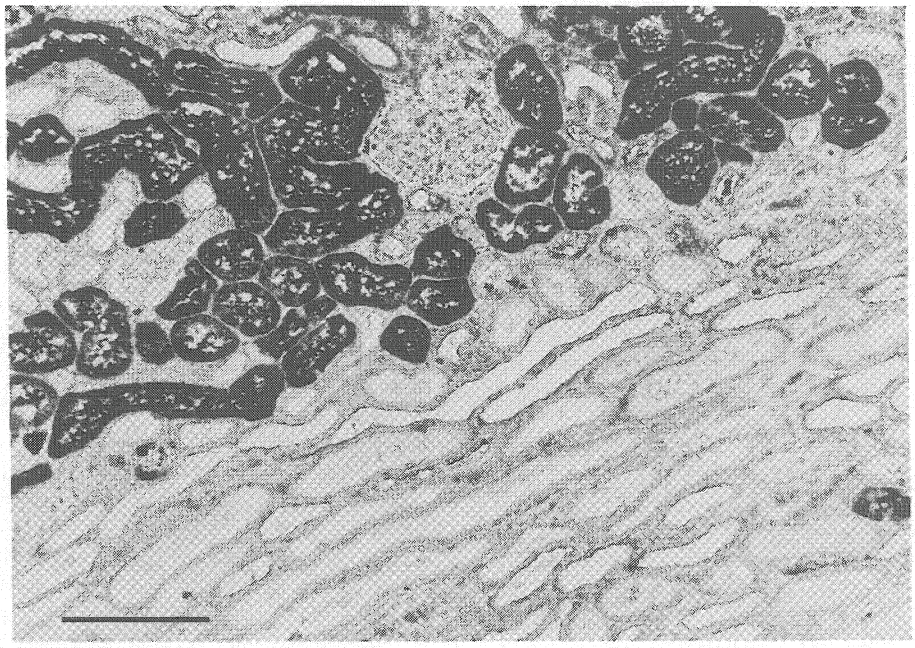

Fig. 4. Immunoperoxidase staining of the cortex of human kidney using antiserum against human kidney FABP A. Only the proximal convoluted tubules are clearly positive. The section was counterstained with haematoxylin. Bar: 200 μm.

meter react moderately and slightly, respectively. In m. soleus and heart, fibers react uniformly for FABP and for succinate dehydrogenase and other oxidative enzymes. Capillary endothelial cells in m. quadriceps react with the anti-heart FABP antiserum (Fig. 3, inset). Smooth muscle cells of blood vessels in rat heart do not stain. Rat heart and quadriceps do not significantly react with anti-liver FABP serum. Human muscle shows a stronger staining with anti-muscle FABP serum on fibers, which are not positive with anti-fast myosine.

Rat kidney shows with anti-heart FABP serum a clear staining of distal straight tubules in the inner stripe of the outer medulla [20]. The cortex shows a more diffuse staining. Antiserum against human kidney FABP A gave a clear specific staining of the proximal convoluted tubules of human kidney; the other tubules and the glomeruli were weakly positive or negative (Fig. 4). With anti-human liver FABP an almost similar distribution of staining was observed as with anti-kidney FABP A, another indication for a large degree of homology of these FABPs.

There is a discrepancy in literature, if FABP is really bound to or present in mitochondria or other cell organelles of heart and liver [3, 7, 9, 27, 28]. This may relate to the used procedures of cell fractionation or assay. This organelle-bound fraction is, however, much less than those reported for the phospholipid transfer proteins [29].

Regulatory and functional aspects of FABP types

The regulation and turnover of FABP types appear to differ [1] markedly. Liver FABP is more responsive to various physiological changes than other FABP types [1]. Postnatal development, sex and clofibrate treatment influence differently liver and intestinal FABPs and their mRNAs [31]. Postnatal development and experimental hypertension influenced the renal FABP type in rat kidney, but not the heart type [8]. The FABP content appears to be markedly influenced by contractile activity in rat muscle [31]. Different genes located on different chromosomes are involved in the encoding of the liver, intestinal and heart FABP types with a tissue-specific expression [14, 32].

Functional properties of FABP types may markedly differ in vivo in view of their variations in ligand-binding specificity and characteristics. The presence of 2 FABP types in the enterocyte suggests differences in function [22]. *In vitro* studies of liver and heart FABPs have not yet shown clear differences in their transport of fatty acid [33]. The various types may, however, form an adaptation to distinct cellular environments and functions: with respect to specific metabolic needs, for targeting of ligands to specific subcellular systems or for protection of cellular membranes or enzyme systems.

In conclusion, FABPs show in comparison to other lipid-transfer or -binding proteins an unique, still increasing number of types, which are partially tissue-specific. They show differences in chemical structure, physicochemical characteristics, ligand specificity, interaction with ligands, regulation and even chromosomal location. The functional significance of the occurrence of different FABP types is, however, unclear. Structural mutants or deficiencies of a FABP type have not yet been reported. Use of cDNA probes for studies on expression, site-directed mutagenesis and transfection may help to get more insight into the structure and function of the various FABP types.

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Fatty acid oxidation capacity and fatty acid-binding protein content of different cell types isolated from rat heart

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Key words: fatty acid oxidation, fatty acid-binding protein, ELISA, cardiomyocytes, endothelial cells, fibroblast-like cells, rat heart

Summary

Heart tissue contains appreciable amounts of fatty acid-binding protein (FABP). FABP is thought to play a crucial role in the transport of fatty acids from the cellular membrane to the intracellular site of oxidation and also, in case of endothelial cells, in the transfer of fatty acids from the vascular to the interstitial compartment through the endothelial cytoplasm. The present study was designed to delineate a possible quantitative relationship between the capacity of different cell types in the heart to oxidize fatty acids and the presence of FABP. Palmitate oxidation capacity, measured in homogenates of cells isolated from adult rat hearts, was 2 nmol/min per mg tissue protein in freshly isolated cardiomyocytes (CMC), but only 0.09 and 0.31 nmol/min per mg tissue protein in cultivated endothelial (CEC) and fibroblast-like cells (CFLC), respectively. Palmitate oxidation rates were closely related to the cytochrome C oxidase activity and, hence, to the mitochondrial density in the cells under investigation. In CMC the content of cytosolic H-FABP (H-FABP$_c$) was about 4.5 μg/mg tissue protein. However, in CEC and CFLC the FABP content was less than 0.01 and 0.004 μg/mg tissue protein, respectively, corresponding to at maximum 0.2% of the FABP content of CMC. These findings indicate a marked difference between CMC and non-myocytal cells in the heart regarding their capacity to oxidize fatty acids, and a marked disproportion between the fatty acid oxidation capacity and immunochemically determined FABP content in both CEC and CFLC. The functional implication of these observations remains to be elucidated.

Introduction

Although the heart can utilize a variety of substrates to fulfill its energy demands, fatty acids are preferred under normal conditions [1]. Since endogenous *de novo* synthesis of fatty acids is limited, these substances are mainly derived from the blood. Cardiomyocytes, occupying about 75% of cardiac tissue volume, are considered to be the principal group of fatty acid consuming cells in the heart, although coronary endothelial cells are also capable of fatty acid oxidation [2].

The exact mechanism underlying the transport of fatty acids from the vascular compartment to the mitochondrial site of consumption in the cardiomyocytes is incompletely understood [3]. It has been hypothetized that fatty acids cross the endothelial cell through the luminal cell membrane, endothelial cytoplasm and abluminal cell membrane, then diffuse through the interstitial space towards the myocytal membrane (sarcolemma), and finally cross the sarcolemma and myocyte cytoplasm to reach the mitochondrial outer membrane, where activation of the fatty acyl moiety occurs [4].

Fatty acid-binding proteins (FABPs) are thought

to be involved in both the transendothelial and the intramyocytal transport of fatty acids [3, 5]. The FABPs belong to a family of relatively low molecular weight proteins, are abundantly present in most cell types and show a high affinity for long-chain fatty acids [6]. Recently it has been shown that the fatty acid-oxidizing capacities of several tissues run in parallel with their FABP contents [7].

The FABP type present in the cytosolic fraction of homogenized rat hearts (H-$FABP_c$) has been extensively characterized and quantified [8, 9]. Information on the cellular distribution of H-$FABP_c$ in the heart is scanty. Immunocytochemical studies have shown that bovine and rat cardiomyocytes contain H-$FABP_c$ [10, 11], whereas considerable staining was also observed in rat cardiac endothelial cells *in situ* [10]. However, reliable quantitative data are lacking. The present study was conducted to quantify H-$FABP_c$ in various cell types isolated from adult rat heart, including cardiomyocytes (CMC), endothelial cells (CEC) and fibroblast-like cells (CFLC). The content of H-$FABP_c$ was related to the capacity to oxidize fatty acids by these particular cardiac cell types.

Experimental procedures

Isolation and cultivation of cells

Cells were isolated, under sterile conditions, from the hearts of male adult Wistar rats, by a modification of the methods of Piper *et al.* [12] and Diglio *et al.* [13]. Hearts were excised from anesthetized rats and placed in ice-cold modified Krebs Ringer buffer (MKR) containing (in mM): NaCl, 115: KCl, 2.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 10; 2-(4-(2-Hydroxy Ethyl)-1-Piper-azinyl)-Ethanesulfonic acid (HEPES), 10; glucose, 11; taurine, 4; pH was set at 7.4 and the buffer solution was gassed with 95% O_2, 5% CO_2. The aorta was cannulated and perfused with MKR according to the Langendorff technique at 37° C at a constant perfusion flow of 10 ml/min for 10 min. After this washing period the heart was perfused with 50 ml MKR containing 25 μM $CaCl_2$ and 0.06% collagenase in a recirculating manner. Endothelial cells (CEC) were collected from the recirculation medium according to Diglio *et al.* [13]. Subsequently, bloodvessels, atria and fibrous tissue were removed from the heart. Thereafter, ventricles were chopped and incubated in 20 ml fresh collagenase solution with 1% bovine serum albumin (BSA) (essentially fatty acid free) for 15 min under gently pipetting the suspension [12]. The solution was filtered through a 200 μm nylon sieve. Cells were centrifuged 90 s at 25 g or given 10 min time to settle down at room temperature.

The formed pellet consisted predominantly of cardiomyocytes (CMC). This fraction was purified for cultivation. The supernatant contained fibroblasts, cardiomyocytes, smooth muscle-, endothelial- and blood cells.

The myocyte pellet was washed three times in MKR with increasing concentrations of calcium (0.2, 0.5 and 1 mM, respectively). Cells were collected by gravity at room temperature. The final pellet was resuspended in myocyte culture medium (M199, 2 mM L-glutamine, 13 mM $NaHCO_3$, 10 mM HEPES, 50 U/ml penicilline, 50 μg/ml streptomycine, 4% foetal calf serum (FCS)) and plated on tissue culture dishes. The dishes were precoated overnight with myocyte culture medium. After 4 hours of incubation at 37° C, in a humidified 95% air/5% CO_2 stove, medium was changed while serum was omitted. More than 95% of the attached cells were non-contracting, rod-shaped and excluded trypan blue. Only a few non-myocytes were present which did not proliferate in serum free medium.

The solutions containing endothelial cells and mixed cells, respectively, were each centrifuged twice with endothelial culture medium (40% M199, 40% RPMI 1640, 20% FCS, supplemented with 2 mM L-glutamine, 13 mM $NaHCO_3$, 10 mM HEPES, 50 μg/ml gentamicine, 2.5 μg/ml fungizone, 20 U/ml heparine and 4 ml/l growth factor prepared from bovine brain according to Maciag *et al.* [14]. The cells were resuspended in culture medium and plated on fibronectine (100 μg/ml) precoated Falcon dishes. Primary cultures were provided with fresh culture medium every day, passaged cultures every 2–3 days. When almost confluent CEC were purified by removing non-endo-

thelial cells under the microscope. Both CEC and mixed cells preparations, of which in primary culture 50–75% had a fibroblast-like (CFLC) appearance, were treated with phosphate buffered saline (PBS) containing 0.67 mM ethylenediaminetetraacetate (EDTA) and 1.25 mg/ml trypsin to detach the cells and subcultivated in endothelial culture medium.

Characterization of cells

The isolated myocytes were checked for cell morfology and trypan blue exclusion. Over 95% of attached cells were rod shaped.

The primary cultures of fibroblast-like and endothelial cells were tested for the uptake of 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indocarbocyanine-perchlorate-Acetylated Low Density Lipoprotein (DilAcLDL) [15]. Cell cultures in 96 wells plates were incubated with 10 μg DilAcLDL/ml culture medium at 37° C for four hours. The medium was renewed and the cells were studied in a Zeiss fluorescence microscope equiped with appropriate barrier filters. For immunofluorescence microscopy cell solutions were plated on 96-wells plates and grown to confluency. Cell monolayers were fixed in 3% paraformaldehyde and treated with 0.05% Nonidet P40 to permeabilize the membranes as described by Laurila *et al.* [16]. After washing three times in PBS, the cells were incubated either with rabbit anti human-Von Willebrand Factor (FVIII/VWF) [17] diluted 1 : 100 in PBS + 1% BSA + 0.1% NaN_3, or with RECA1 (a rat endothelial cell antibody) diluted 1 : 500 in PBS + 1% BSA + 0.1% NaN_3 in a humidified atmosphere at 37° C for 30 min. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated swine anti rabbit-IgGs or FITC-conjugated rabbit anti mouse-IgGs. The cells were washed and mounted in glycin buffered glycerol (pH 8.6) for microscopic observation.

Experimental conditions and assay techniques

Cultured CEC and CFLC between passage two and six, and primary CMC were washed three times with prewarmed Hanks' Balanced Salt Solution containingg 10 mM HEPES (HBSS). Cells (approximately 1 mg protein in 300 μl HBSS per dish) were scraped from the dishes with a rubber policeman. The homogenization final fluid contained 250 mM sucrose and 1 mM EDTA. This solution was homogenized in a Potter-Elvehjem homogenizer, using successive pestle clearances of 0.1 and 0.05 mm.

The oxidation of 120 μM [1-^{14}C]palmitate bound to albumin (molar ratio 5 : 1) was measured at 37° C for 30 minutes with 0.2 to 0.3 mg cellular protein per incubation. Oxidation rates were calculated from the radioactivity of the formed $^{14}CO_2$ and ^{14}C-labeled acid-soluble intermediates as described by Glatz *et al.* [18]. Protein content was determined by the method of Lowry *et al.* [19]. Cytochrome C oxidase activity of the homogenates was measured at 25° C as described [20].

The H-FABP$_c$ content in the different cell homogenates was measured by enzyme-linked immunosorbent assay (ELISA) using polyclonal rabbit antibodies raised against rat H-FABP$_c$. After centrifugation (10000 g, 5 min) of the homogenates, the supernatants were directly coated to PVC microtiterplates and quantified by comparing them with a series of H-FABP$_c$ standards in a concentration range of 2 to 20 μg/l. After incubation with anti-H-FABP$_c$ antibodies and goat anti rabbit-IgG/HRPO, the oxidation rate of o-phenylenediamine was monitored in a Flow Multiscan plus MKII.

Materials

The culture media RPMI 1640, M199 and the trypsin solution were purchased from Flow (Irvine, Scotland), FCS from Boehringer (Mannheim, FRG) and Seralab Ltd. (Sanbio, Uden, the Netherlands). Collagenase (CLS) was obtained from Cooper Biomedical (Worthington). BSA (essentially fatty acid free) V was obtained from Sigma (St Louis USA) and BSA V from Boehringer (Mannheim, FRG).

The Falcon tissue culture dishes and microtiter plates were obtained from Becton Dickinson BV

(Etten-Leur, The Netherlands). FITC conjugated antibodies were purchased from Dakopatts (Copenhagen, Denmark), goat anti rabbit PO/HRPO was obtained from Nordic (Tilburg, the Netherlands). DilAcLDL from Biomedical Technologies Inc. (Stoughton, USA). RECA 1 was a gift from Dr A Duijvestijn (University of Limburg, Department of Immunology, The Netherlands). Fibronectin and rabbit polyclonal anti-Von Willebrand protein IgG was obtained from the Central Laboratory of the Netherlands Red Cross Transfusion Service (CLB Amsterdam).

Results

The cardiomyocytes used in this study were kept in culture during four hours after isolation. They were mechanically at rest. Microscopical evaluation showed that over 95% of the cells were rod-shaped and excluded trypan blue. The CEC preparation consisted mainly of endothelial cells. When during the first culture non-typically growing cells were observed microscopically, these particular cells were removed from the plate. The remaining cells were allowed to grow to confluency. This is a slight modification of the method as described by Diglio *et al.* [13]. The fresh CFLC preparation was not pure. In addition to fibroblasts several other cell types were present, including endothelial cells. The latter cell type could be easily recognized owing to its typical growth pattern. In the order of 15 to 25% of primary CFLC culture consisted of endothelial cells. The proportion of endothelial cells rapidly decreased during subcultivation. They were most likely overgrown by the fibroblast-like cells. After passage two, endothelial cells could no longer be recognized in the CFLC preparation.

Endothelial cells (CEC) were positively identified by three independent techniques. First by the uptake of DilAcLDL. Second by staining of the Factor VIII/Von Willebrand factor, known to be present in vesicles in the cytoplasmic compartment of endothelial cells, and stainable with the use of a specific antibody. Third, by staining of the endothelial cell membrane with the use of a specific antibody raised against rat endothelial cells. All three techniques positively identified the cultivated CEC as endothelial cells (Table 1). The CFLC preparation, at passage two or higher lacked endothelial cells. On the basis of morphological criteria and their typical growth patterns, the cells in the CFLC preparation were best identified as cardiac fibroblasts-like cells (Table 1). Fibroblast-like cells formed a monolayer if confluent, in post-confluent state they overgrew each other and had a more spindle-like appearance.

Palmitate oxidation was measured radiochemically in homogenates of the cells at concentrations of substrate, coenzymes and cofactors that gave maximal oxidation rates [18]. The oxidation capacity of cardiomyocytes (CMC) was 2.31 ± 0.31 nmol/min per mg tissue protein. However, endothelial cells (CEC) and fibroblast-like cells (CFLC) had a considerably lower oxidation capacity (Table 2). The distribution of the activity of the mitochondrial marker enzyme cytochrome C oxidase, measured in the homogenates of CMC, CEC and CFLC (Table 2), indicated that the mitochondrial density was highest in CMC.

Table 1. Characterization of different cell types isolated from adult rat heart

Cells	Morphology	Identification		
		Factor VIII/VWF	Rat endothelial cell antibody	DilAcLDL
CMC	rod shaped	nt	nt	nt
CEC	clonal growth	+	+	+
CFLC	non-clonal growth monolayer	−	−	−

CMC = cardiomyocytes; CEC = cardiac endothelial cells; CFLC = cardiac fibroblast-like cells; nt = not tested. The methods used to identify the various cell types are described in detail in the methods section; CEC and CFLC were used between passage two and six.

The content of H-FABP$_c$ was found to be 4.5 ± 1.4 μg/mg tissue protein in CMC, and below 0.008 μg/mg tissue protein in CEC and CFLC (Table 2). The detection level of the FABP-assay was 0.002 μg/mg tissue protein.

Discussion

The present findings indicate that the main locus of cardiac fatty acid oxidation is the cardiomyocyte. Assuming that in the order of 90% of the cardiac protein is of cardiomyocytal origin, cardiomyocytes contribute by about 98% to the total cardiac fatty acid oxidation capacity. This notion supports the observations made in isolated coronary microvascular cells as reported earlier [2].

On the basis of the specific activity of the mitochondrial marker enzyme cytochrome C oxidase it can be concluded that the contents of mitochondria in endothelial (CEC) and fibroblast-like cells (CFLC) are considerably less than in cardiomyocytes (CMC). Figure 1 indicates that the capacity to oxidize fatty acids is closely related to the activity of cytochrome C oxidase and hence, mitochondrial density of individual cell preparations.

The bulk of fatty acids used for oxidative degradation is supplied to the heart from the vascular compartment. Fatty acids are assumed to cross the endothelium, separating the vascular and interstitial compartment, directly through the cytoplasm of the endothelial cells. Since fatty acids have an extremely low aqueous solubility, intracellular carriers are required for their transport from the luminal to the abluminal site of the endothelial cell. Analogously, carrier moieties are also needed to transport fatty acids from the site of entry, i.e. the myocyte plasma membrane, to the mitochondria, which are the main locus of fatty acid oxidation in heart tissue. FABP has been proposed to fulfill this fatty acid-carrier role [5, 10]. In this respect, it should be mentioned that for a number of organs a quantitative relation exists between the fatty acid oxidation capacity and the cytosolic capacity to bind fatty acids [7], suggesting a close functional relationship between fatty acid binding, most likely by a specific cytoplasmic fatty acid-binding protein (FABP$_c$), and mitochondrial fatty acid oxidation.

In concert with this notion, in isolated CMC a relatively high fatty acid oxidation capacity was observed to be associated with a relatively high content of FABP. However, a divergent picture emerges from data obtained in isolated cardiac endothelial and fibroblast-like cells. Although, on the basis of total cellular protein, the oxidation capacities are 3–15% of that of CMC, the contents of FABP are even one order of magnitude lower, being approximately 0.1% of that measured in CMC.

The latter finding disagrees with earlier observations by Fournier and colleagues [10], who calculated from electron microscopic immunocytochemical studies that the level of FABP in the cardiac endothelial cell is about half of that in the cardiomyocyte.

A possible explanation for this striking discrepancy is that CEC contain FABP(s) to which the polyclonal antibody (raised against rat H-FABP$_c$), as used in the present study, does not react to any significant extent. When this is true, at least two

Table 2. Palmitate oxidation rate, cytochrome C oxidase activity and H-FABP$_c$ content in various cell types isolated from adult rat heart

Cell type	Palmitate oxidation rate (nmol/min per mg tissue protein)	Cytochrome C oxidase activity (U/mg tissue protein)	H-FABP$_c$ content* (μg/mg tissue protein)	Ratio H-FABP$_c$ content to palmitate oxidation rate (μg · min/nmol)
CMC (n = 4–6)	2.31 ± 0.31	7.36 ± 2.42	4.5 ± 1.4	2.1 ± 1.6
CEC (n = 6)	0.09 ± 0.05	0.28 ± 0.10	0.0078 ± 0.0040	0.096 ± 0.055
CFLC (n = 5)	0.31 ± 0.19	0.52 ± 0.12	0.0033 ± 0.0036	0.009 ± 0.006

Data are expressed as means ± SD of the indicated number of the cell preparations.
* Assayed in 10,000 g supernatants of homogenates of the cell preparations.

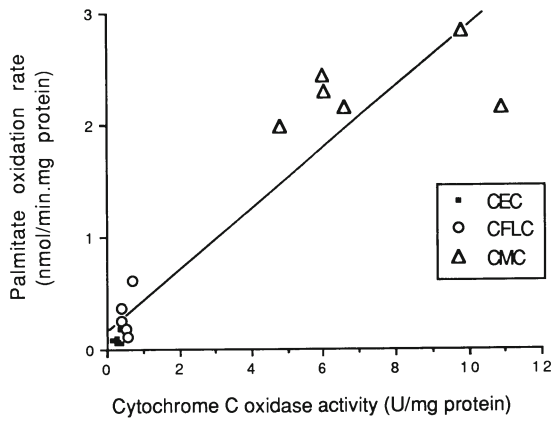

Fig. 1. Relation between palmitate oxidation rate and cytochrome C oxidase activity in cardiomyocytes (CMC), endothelial (CEC) and fibroblast-like cells (CFLC) isolated from adult rat heart. Data for individual cell preparations are given. The calculated regression coefficient is 0.94. Values are expressed per total tissue protein.

different FABPs have to be present in the heart, one localized in the cardiomyocytes and a second one in endothelial and/or fibroblast-like cells. Experiments are under way to measure the capacity of a cytosolic protein preparation from isolated CEC to bind fatty acids, so as to identify the possible presence of an endothelial-specific FABP. Alternatively, with CEC and CFLC the recovery of H-FABP in the supernatant after centrifugation of the homogenate could be highly incomplete, e.g. due to protein binding to membrane structures. Finally, during culture CEC and CFLC could possibly lose their ability to produce FABP.

Assuming that extrapolation of the present *in vitro* findings on the virtual absence of myocyte-FABP in CEC to cardiac endothelial cells *in situ* is allowed, the lack of specific fatty acid-binding proteins in this particular cell type will have important physiological consequences for the mechanism of transendothelial transport of long-chain fatty-acids and their transfer from the cell membrane to the mitochondrial site of oxidation inside the endothelial cells.

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Localization of liver fatty acid-binding protein and its mRNA in the liver and jejunum of rats: an immunohistochemical and *in situ* hybridization study

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Key words: liver fatty acid-binding protein, immunohistochemistry, *in situ* hybridization, liver, jejunum, rat

Summary

The localization of liver fatty acid-binding protein (L-FABP) and its mRNA in the liver and jejunum was examined in normal and 3-day-fasted rats by means of immunohistochemistry using a specific antibody to L-FABP and *in situ* hybridization using a synthetic oligonucleotide complementary to L-FABP mRNA as probe. In the liver from normally fed rats, the signal for L-FABP mRNA in hepatocytes was distributed throughout the lobule, with higher intensity in the periportal than in the centrolobular region. After a 3-d fasting, the mRNA signal declined in intensity throughout the lobule, in accordance with the result of Northern blot analysis. Immunohistochemistry for L-FABP showed intralobular patterns of immunoreactivity similar to those of the mRNA signal in both fed and fasted animals. In the jejunum from fed rats, L-FABP-mRNA signal was abundant in the absorptive epithelial cells lining the lower two-thirds of villus and less abundant in the villus tip cells, while the intensity of L-FABP immunoreactivity remained high in the latter cells. Fasting brought about a downward shift of the mRNA signal to an area including the upper half of the crypt and the lower portions of villus, with decreased intensity in the rest of the villus. Immunohistochemistry also showed a downward extension of the immunoreactivity into the upper crypt area. The present results suggest that *in situ* hybridization is a useful tool to analyze regulations of the expression of L-FABP gene in the digestive organs in association with epithelial cell migration and dietary condition.

Introduction

Fatty acid-binding proteins (FABPs) comprise a group of small molecular weight cytosolic proteins that bind fatty acids and their acyl CoA derivatives [1]. Rat liver fatty acid-binding protein (L-FABP), with MW 14000, was originally isolated from the liver [2, 3]. It has been shown to be expressed predominantly in the liver and intestine, on the basis of the biochemical studies such as immunochemical detection of L-FABP in the cytosol preparations [4, 5] and Northern blot analysis of the mRNA preparations [6, 7]. Several immunohistochemical studies have further determined the cell specificity and intracellular localization of L-FABP expression in the liver [8–10] and intestine [11–13]. However, histochemical approach to the localization of mRNA for L-FABP has never been attempted. In the present study, we performed *in situ* hybridization histochemistry to localize L-FABP mRNA in the liver and jejunual sections of rats and examined the effect of fasting on the distribution pattern of L-FABP mRNA. For this purpose, an synthetic oligonucleotide probe of 30-base length which was complementary in sequence to the 5′-noncoding region of L-FABP mRNA was used.

The result was compared with that of the immunohistochemistry for L-FABP.

Methods

Animals

Male Wistar rats aged 8 weeks (about 250 g body weight) were divided into two groups of eight animals. One group was fed ad libitum with standard rat chows and water, and the other was fasted for 3 days with free access to water. All animals were sacrificed at 11.00 AM.

Oligonucleotide probe

A 30-mer deoxyribonucleotide probe (5′-GAG GTT TCC TTT CCC AGC TGC CAC CAA CAG-3′), which was complementary to a portion of the 5′-noncoding sequence of rat L-FABP mRNA [6], was synthesized using a Beckman System 1-plus DNA Synthesizer. For methodological control, a template oligonucleotide complementary to the probe was also synthesized.

Northern blot analysis

Three animals from each group were sacrificed by decapitation and the total RNA was isolated from the stomach, liver (middle lobe) and jejunum (proximal half of the small intestinal portion from the ligament of Treitz to the ileocoecal valve) using a guanidine thiocyanate method [14]. The RNA samples (20 μg) from each organs were denatured by glyoxal, separated by electrophoresis in 1.0% agarose gel and subsequently transfered to nitrocellulose filter according to Thomas [15]. The oligonucleotide probe was labeled at the 3′-terminus with terminal transferase (Boeringer) using [γ-^{32}P]-dCTP (Amersham) as described [16]. It was then hybridized with the filters in a mixture containing 1 M NaCl, 10 × Denhardt's solution (1 × Denhardt's = 0.02% Ficoll, 0.02% polyvinyl pyrrolidon and 0.02% bovine serum albumin), 10 mM EDTA, 0.1% sodium lauroyl sarcosinate (Sarkosyl) and sheared salmon sperm DNA (250 μg/ml) in 0.1 M Tris-HCl (pH 7.5) at 55° C overnight. After washing at the same stringency as in hybridization, the filters were exposed for autoradiography using Kodak X-Omat AR film with intensifying screens.

Preparation of tissue sections

Five animals from each group were anesthetized intraperitoneally with sodium pentobarbital and fixed by perfusing transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Tissue specimens were excised from the liver (middle lobe) and jejunum (segment at proximal one-third of small intestine) and immersed in the same fixative for an additional 4 h. They were subsequently rinsed overnight in 30% sucrose and snap-frozen in liquid nitrogen. Sections (15 μm thick) were made with a cryostat and mounted on glass slides coated with 1% gelatin/0.5% chrome alum.

In situ *hybridization*

The oligonucleotide probe was labeled by 3′-tailing to a specific activity of 1–2 × 10^8 cpm/μg using [α-^{35}S]-dCTP (New England Nuclear). The hybridization procedure was essentially as described previously [17, 18]. Briefly, the sections were incubated with a prehybridization mixture containing 50% deionized formamide, 4 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 1 × Denhardt's solution, 2% Sarkosyl, 20 mM 2-mercaptoethanol and 250 μg/ml of heat denatured salmon sperm DNA in 0.1 M sodium phosphate buffer (pH 7.2), and then with a hybridization mixture containing 5 × 10^5 dpm of the labeled L-FABP probe. For the methodological control experiment, an excess amount (50 times that of the labeled probe) of the unlabeled template oligonucleotide complementary to the probe was added to the mixture. After incubation for 16 h at 37° C, the sections were washed thoroughly in 2 × SSC at room temperature, followed by 1 × SSC plus 0.1% Sarkosyl at 37° C. After dehydration, the slides were dipped

in Kodak NTB2 nuclear track emulsion and incubated for 10 days at 4° C for autoradiographic exposure.

Immunohistochemistry

The IgG fraction of the rabbit antisera against purified rat L-FABP (identical to DE-II fraction of Z-protein, Ref 3) was prepared as described previously [9, 13]. This antibody was identified as monospecific to L-FABP by both Ouchterlony double diffusion method and Western blotting method. As control, anti-L-FABP antibody that had been absorbed with L-FABP was used. For light microscopic immunohistochemistry, the sections were incubated overnight at room temperature with the anti-L-FABP or control antibody solution in PBS (4.7 μg IgG/ml). Following treatment of sections with swine anti-rabbit IgG antibody (Dakopatts), the sites of immunoreaction were visualized with the peroxidase-antiperoxidase (PAP) method according to Sternberger [19].

Results

Northern blot analysis of RNA samples

First, the oligonucleotide probe was labeled with ^{32}P and used for a Northern blot hybridization to detect L-FABP mRNA in the total cellular RNA samples from the stomach, liver and jejunum (Fig. 1). In the RNAs of liver and jejunum from fed animals, a single RNA band with an approximate size of 700 bases, which corresponded to L-FABP mRNA on agarose gel [6, 7], hybridized to the probe, while hybridization was barely detectable with the stomach RNA. These results indicated that the probe detected L-FABP mRNA specifically. By densitometric tracing of the autoradiograph, the relative abundance of L-FABP mRNA to total RNA was 1.7 times higher in the jejunum than in the liver. After a 3-d period of fasting, the relative abundance of L-FABP mRNA declined to 25% (liver) or 46% (jejunum) that of fed control (mean of 3 separate experiments).

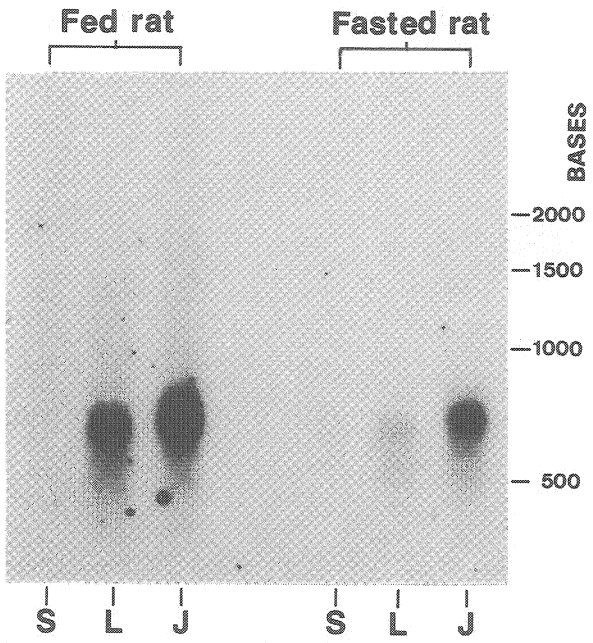

Fig. 1. An autoradiograph showing Northern blot hybridization of total cellular RNA from the stomach (S), liver (L) and jejunum (J) of fed and 3-d-fasted rats to an oligonucleotide probe complementary to L-FABP mRNA. Twenty μg of RNA was denatured, electrophoresed on an agarose gel, transferred to nitrocellulose and hybridized to the ^{32}P-labeled probe. The scale for RNA size (bases) was determined by co-migration of E. coli and bovine rRNAs.

Liver sections

When the liver sections from rats fed ad libitum were incubated with the ^{35}S-labeled oligonucleotide probe, autoradiographic silver grains representing the hybridization signal for L-FABP mRNA were localized in the hepatocytes and not detected in the non-parenchymal cells associated with the blood vessels. Although the mRNA signal was distributed throughout the lobule, its intensity was substantially lower in the area surrounding the central vein than in the periportal area (Fig. 2a). When an excess amount of 'template' oligonucleotide which was complementary to the probe was added to the hybridization mixture, the silver grains due to the labeled probe was completely abolished, indicating that the signal was specific to L-FABP mRNA. In immunohistochemistry for L-FABP, all hepatocytes were immunoreactive, but

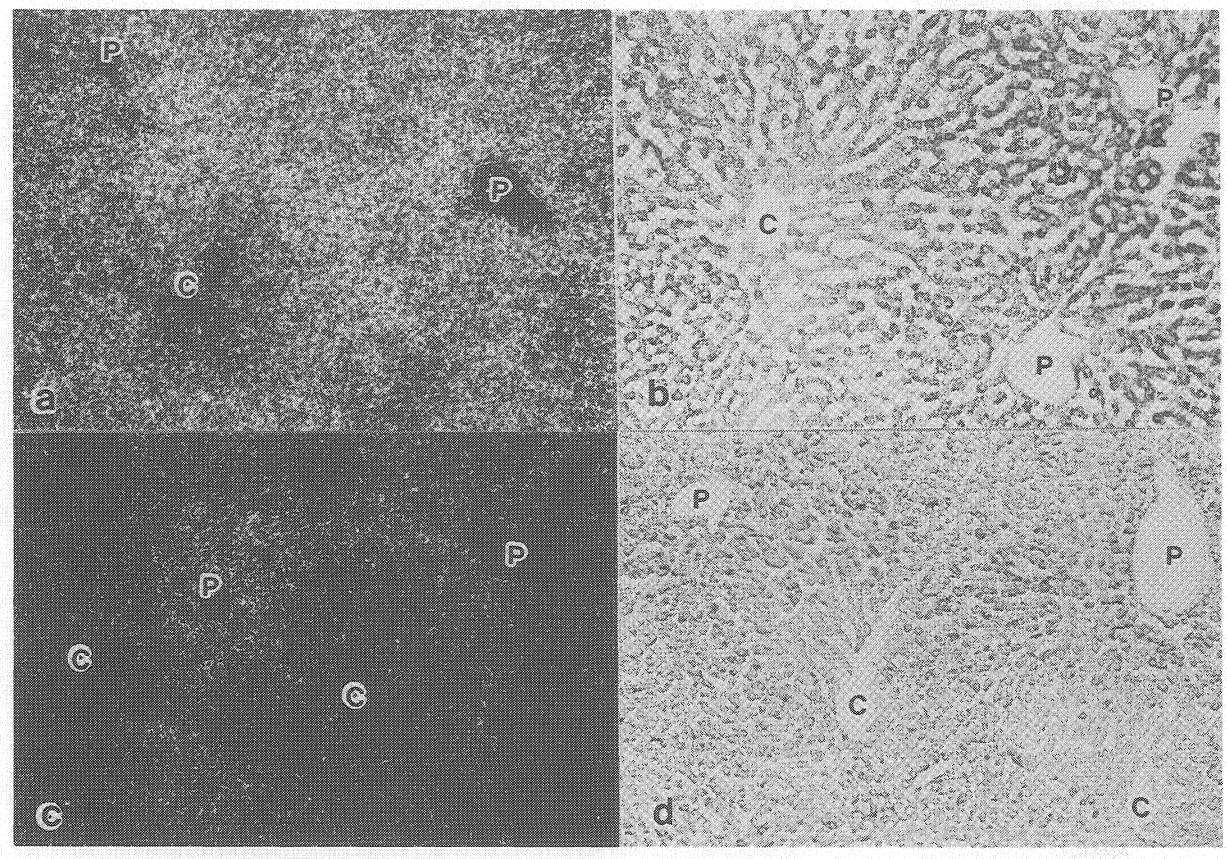

Fig. 2. Autoradiographs in dark field showing in situ hybridization for L-FABP mRNA (Figs 2a, c) and light micrographs showing immunohistochemistry for L-FABP (Figs 2b, d) in the liver sections from fed (Figs 1a, b) and 3-d-fasted (Figs 1c, d) rats. Note the concentrations of both silver grains and immunostaining lower in the hepatocytes surrounding the central vein (C) than in those around the portal tract (P). Also note an overall decrease in the concentration of silver grains and immunostaining in fasted rats. × 80.

the staining intensity showed a decreasing gradient from the portal to centrovenular direction, in agreement with the distribution pattern of the mRNA (Fig. 2b). When control antibody preabsorbed by L-FABP was used, no immunostaining was observed in any cells and structures in the liver, indicating that the immunoreactivity was specific to L-FABP.

After a 3-d period of fasting, the silver grains representing L-FABP mRNA declined substantially in number in all hepatocytes, although a slight portal-centrovenular decreasing gradient of signal intensity was still discernible (Fig. 2c). Immunohistochemistry also showed an overall decline as well as a subtle intralobular gradient in the intensity of L-FABP immunoreactivity similar to those of the mRNA signal (Fig. 2d).

Jejunal sections

In in situ hybridization of the jejunal sections from fed rats, the L-FABP-mRNA signal was detected in absorptive epithelial cells of the villi but was absent in the crypt epithelial cells, making a distinct lower boundary of the labeled area at the hight of villus-crypt junction (Fig. 3a). The silver grains were particularly abundant in the lower two-thirds of villus while decreased in number in the villus tip area. When the sections were immunostained with anti-L-FABP antibody, all absorptive epithelial

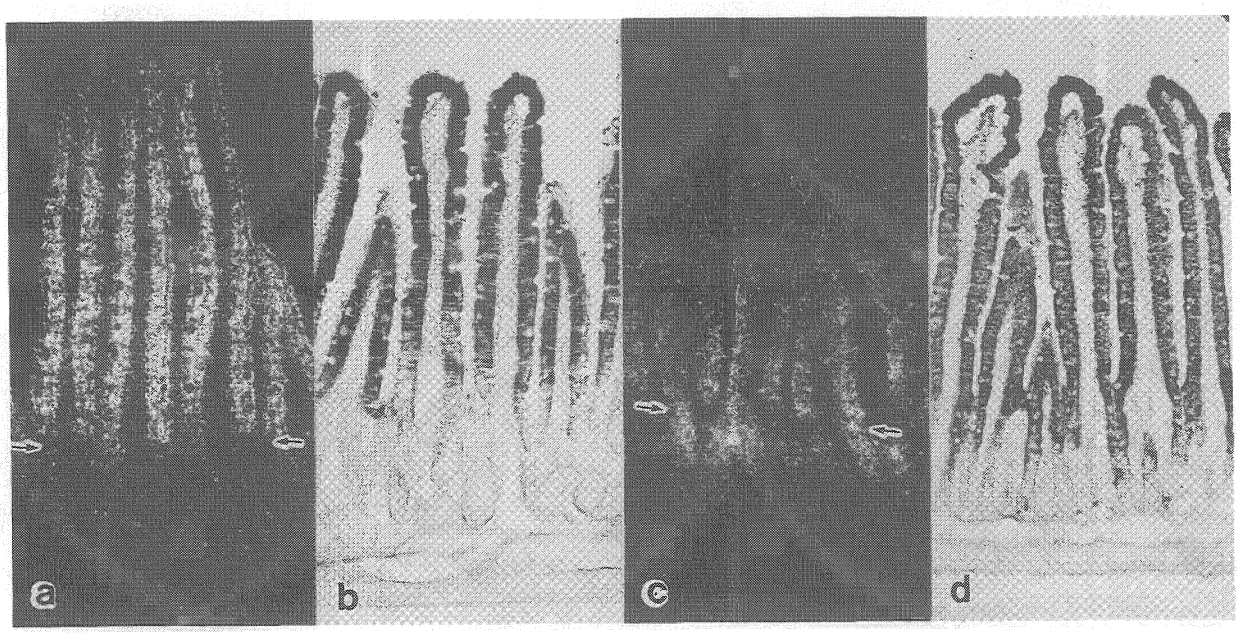

Fig. 3. Autoradiographs in dark field showing in situ hybridization for L-FABP mRNA (Figs 3a, c) and light micrographs showing immunohistochemistry for L-FABP (Figs 3b, d) in the jejunal sections from fed (Figs 3a, b) and 3-d-fasted (Figs 3c, d) rats. Note the higher concentration of silver grains in the lower two-thirds of villus (Fig. 3a) or in the upper half of crypt and lower one-third of villus (Fig. 3c). Also note the higher intensity of immunostaining in the entire villus (Fig. 3b) or in the upper half of crypt as well as entire villus (Fig. 3d). Arrows indicate the junction between crypt and villus. × 80.

cells lining the villus were immunoreactive, with an equal or even higher intensity of reactivity in the villus tip cells than in the remainder (Fig. 3b).

After a 3-d fasting, an intense signal for L-FABP mRNA was observed in the upper half of crypt as well as in the lower one-third of villus. The signal decreased gradually in intensity in the upper portions of villus toward the tip (Fig. 3c). Immunohistochemistry confirmed a downward shift of the lower boundary of immunoreactive cell population into the upper half of crypt, although the intensity of immunoreactivity remained high in the upper portions of villus including the tip area (Fig. 3d).

Discussion

In situ hybridization histochemistry using synthetic oligodeoxyribonucleotide (oligomer) probes has been successfully used to detect mRNAs in various tissues [20–22]. In the present study, an oligomer probe for L-FABP mRNA has revealed the distribution patterns of the mRNA signal in the liver and jejunum. The result in liver is essentially in agreement with that of immunohistochemistry, i.e., the predominance of L-FABP immunoreactivity in the periportal hepatocytes, as already demonstrated by Bass *et al.* [10]. However, in the jejunum, a lower intensity of mRNA signals is in contrast to a higher intensity of immunoreactivity in the villus tip absorptive cells, when compared with the rest of villus cells. This discrepancy may partly be explained by a possibility that the translation rate of L-FABP does not parallel the abundance of L-FABP mRNA from portion to portion of the tissue. The second and more likely explanation for the different distribution patterns of L-FABP and its mRNA is a relatively long turnover time of L-FABP. Since the half-life of L-FABP is estimated to be 3.1 days [23], which is even longer than 2 days required for the crypt epithelial cells to migrate up to the villus tip [24], the protein may accumulate in the villus tip cells in spite of a decreased transcription rate.

In regard to the effect of fasting on the expression of L-FABP, there are reports that the relative concentration of L-FABP to total cellular protein either is unchanged [5] or decreases slightly [23] in the liver, but increases in the small intestine [25] after a 48 to 66-h fast. The present result of Northern blot hybridization has revealed that the relative abundance of L-FABP mRNA to total cellular RNA decreases in both organs after a 3-d fast. Furthermore, the present *in situ* hybridization analysis has revealed the distribution patterns of L-FABP mRNA in both organs after fasting. In the context of the result in liver there is immunohistochemical evidence for a circumscribed localization of L-FABP around the glycogen particles in individual hepatocytes [8], a pattern that is abolished in association with disappearance of glycogen after a 2 to 3-d period of fasting [9]. Therefore, the present result implies a regulation of L-FABP-gene expression coincided with the cytoplasmic glycogen content or distribution. In the liver lobule, the centrolobular hepatocytes are known to loose glycogen faster than the periportal hepatocytes during fasting [26]. However, we cannot determine in the present study, where a single fasting duration of 3 days is adopted, if there is any predominant site for fasting-induced loss of L-FABP mRNA within the liver lobule. Furthermore, a recent report by Bass *et al.* [10] has shown that there is a marked difference between untreated male and female rats in the intralobular distribution of L-FABP immunoreactivity. Perhaps a thorough longitudinal study using fasted rats of both sexes may bring out further information on the fasting-induced suppression of L-FABP-gene expression in the hepatocytes. On the other hand, the fasting-induced expression of FABP gene in the cells lining the upper half of crypt, in terms of both the mRNA signal and immunoreactivity, bears certain implication in regard to the functional role of L-FABP in the jejunum: L-FABP synthesized prior to the maturation of crypt cells into villus absorptive cells may be involved in an increased uptake and utilization of plasma-derived free fatty acids, which are known to rise in concentration after fasting [1], by the crypt epithelial cells.

The present results suggest that *in situ* hybridization is a useful tool to analyze regulations of gene expression that work on relatively abundant and long-lived proteins like L-FABP in a few-day term, such as those associated with epithelial cell migration and dietary condition.

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Amino acid sequence and some ligand binding properties of fatty acid-binding protein from bovine brain*

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Key words: amino acid sequence, bovine, brain, binding

Summary

A fatty acid-binding protein (FABP) from the cytosol of bovine brain was purified by Sephadex G-75 filtration and electrofocusing. The purified protein migrated as a single protein band in 15% polyacrylamide gel electrophoresis with an apparent molecular mass of 14.7 kDa. To ascertain that the purified protein was a FABP, it was submitted to fatty acid-binding tests. Oleic and palmitic acids bound to brain FABP but this was not the case for palmitoyl CoA. By Scatchard analysis the ligand binding values were: Kd = 0.28 μM, Bmax (mol/mol) = 0.6 for oleic acid and Kd = 0.8 μM, Bmax (mol/mol) = 2.1 for palmitic acid. The complete amino acid sequence of the brain FABP was determined and a microheterogeneity was observed. Sequence comparison with other FABPs of known sequence and the observed microheterogeneity demonstrated the presence in brain of several homologous FABPs closely related to heart FABP.

Introduction

The presence of FABP in brain was suggested by the observations that fatty acids were potential regulators of neuroactive amino acid transport in synaptosomal systems and by the finding that synaptosomal Na^+-dependent amino acid transport was stimulated by FABPs [1–3]. Moreover, the presence of mRNA encoding FABP was described in brain [4] and partial purification of a FABP from rat brain was already reported by other authors [3, 5]. Because of our interest in brain cytosolic non-enzymic proteins involved in the metabolism of lipophilic compounds, we decided to isolate a FABP from bovine brain. FABPs are distributed in a wide variety of tissues and extensive structural studies of FABPs isolated from different mammalian organs have resulted in the identification of three homologous proteins. They are named liver (L-), intestinal (I-) and heart (H-) FABP based on their primary site of isolation.

In order to understand the structural basis for the multiplicity and functional properties of the FABPs, we determined the primary structure of bovine brain FABP and evaluated its binding characteristics. Our sequence data compared with other FABPs of known sequence demonstrated the presence in brain of several homologous FABPs closely related to heart FABP.

Experimental procedures

The details concerning the purification of brain

* This paper corresponds to a communication at the first international workshop on fatty acid binding proteins (Maastricht, the Netherlands, September 4–5, 1989).

FABP and the experimental conditions of its complete sequence determination were described elsewhere [6].

Materials

Sephadex G-75 fine and superfine were obtained from Pharmacia. Lipidex 1000 was purchased from Packard. Radiolabeled fatty acids and palmitoyl-CoA were obtained from New England Nuclear. Trypsin, chymotrypsin, carboxypeptidase were purchased from Worthington and elastase from Boehringer. The reagents employed for the sequencer were obtained from Applied Biosystems; all other reagents were purchased from Merck or Prolabo.

Purification of bovine brain FABP

Bovine brains were obtained within 2 h death and a soluble brain extract was prepared by homogeneization and centrifugation at 120 000 g. All the purification steps were carried out in the cold and the FABP was purified from soluble brain extract by filtration, delipidation on Lipidex 1000 and electrofocusing. Filtration was performed on Sephadex G-75 fine (3 × 100 cm) in a Tris-HCl, 150 mM NaCl, pH 7.4 buffer. The fractions containing proteins of molecular mass around 15 kDa were dialyzed, concentrated and then submitted to filtration on a Sephadex G-75 superfine column in presence of [^{3}H]oleic acid (10^6 cpm). Radioactivity was determined by liquid-scintillation counting and the fractions containing the FABP bound to the labeled fatty acid were pooled. To remove [^{3}H]oleic acid bound to FABP, the FABP-fatty acid complex was applied to a Lipidex column equilibrated and developed at 37° C with 10 mM potassium phosphate pH 7.4. FABP-containing fractions were submitted to electrofocusing with an LKB 8101 column using carrier ampholytes in the pH range 4–8 in a sucrose density gradient. Electrofocusing was performed at a constant power of 2.5 W.

Analytical polyacrylamide gel electrophoresis

Purity of bovine brain FABP was controlled by analytical 15% polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS) as described by Laemmli [7].

Fatty acid-binding studies

Protein samples (between 1–18 μg) were incubated with radiolabeled fatty acids (50–100 pmol) in 10 mM potassium phosphate pH 7.4. The total volume was 500 μl and after incubation for 10 min at 37° C, the samples were cooled on ice and loaded on 2 ml Lipidex columns. Protein-bound ligand was eluted with 10 mM potassium phosphate pH 7.4 at 4° C. The free ligand was retained and was then eluted with methanol at room temperature. Aliquots of the eluted fractions were assayed for radioactivity by liquid sintillation counting.

Sequence determination

The brain FABP was submitted to trypsin, chymotrypsin and elastase digestions in 0.1 M ammonium bicarbonate with an enzyme/substrate ratio of 1/50. Cyanogen bromide cleavage was performed in 70% formic acid for 48 h at 20° C. The obtained peptides were purified by HPLC using a Browlee RP300 column (4.6 × 22 cm). The purified peptides were submitted to automated Edman degradation in an Applied Biosystems 470 A protein sequencer.

Results

Purification of the bovine brain FABP

An aliquot of the purified brain FABP was submitted to analytical 15% polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Bovine brain migrated as a single protein band with an apparent molecular mass of 14.7 kDa.

Ligand-binding properties

In order to verify that the purified protein was a FABP, it was submitted to fatty acid-binding tests. Oleic, palmitic and stearic acids bound to brain FABP while palmitoyl-CoA did not bind. The apparent K_d and maximum binding capacity Bmax (mol/mol) of oleic and palmitic acids, calculated by Scatchard analysis, are shown in Table 1.

Amino acid sequence

Brain FABP subjected to Edman degradation did not give rise to phenylthiohydantoin derivatives, suggesting a blocked N-terminal amino acid residue.

The C-terminal amino acid was established by digestion with carboxypeptidase A: 0.6 mol Gln/mol protein was characterized. The split peptides obtained by the various cleavage methods were purified by HPLC and led to the determination of the complete amino acid sequence of the brain FABP. The detailed sequence data necessary for the unequivocal determination of the primary structure have previously been published [6] and the complete sequence is shown in Fig. 1. It is noteworthy that for 20 residues, 2 different amino acids were found at the same position in the sequence.

Discussion

Tested in 15% SDS-polyacrylamide gel electrophoresis, bovine brain FABP migrated as a single protein band. As compared with marker proteins, the apparent molecular mass of bovine brain FABP was 14.7 kDa. This is in accordance with the M_r (14 574) calculated from the brain FABP primary sequence and is similar to the M_r described for rat liver FABP (14 184) [8] and bovine heart FABP (14 673) [9].

Table 1. Oleic and palmitic acid binding by bovine brain FABP. 50–1000 pmol of radiolabeled fatty acid was incubated 10 min at 37° C with the purified FABP in a total volume of 500 µl buffer solution. The amount of brain FABP used per assay was 10 µg for oleic acid and 3 µg for palmitic acid. The binding values were derived from Scatchard analysis of individual isotherms.

Acid	Apparent K_d (µM)	Bmax (mol/mol)
Oleic acid	0.28 ± 0.04	0.6
Palmitic acid	0.8 ± 0.12	2.1

The ligand-binding constants of brain FABP are presented in Table 1. Brain FABP seems to have a preference for oleic acid (K_d = 0.28 µM) over palmitic acid (K_d = 0.8 µM). The values are of the same order of magnitude as the K_d described for other FABPs. Indeed, it was reported that L-FABP binds long-chain fatty acids with a K_d of 0.4–0.9 µM [10] and human H-FABP was recently described as binding oleic acid with a K_d of 0.2 µM and palmitic acid with a K_d of 0.6 µM [11]. In our experiments, we observed no binding of palmitoyl CoA to brain FABP. Such an observation is in contradiction with the binding capacities of L-FABP towards this compound. A discrepancy is usually observed in the stoichiometry of ligand binding by FABPs. It has generally been accepted that they bind one mole of fatty acid/mole protein [12–14]. However, Offner *et al.* [15] indicated that both rat L-FABP and rat H-FABP bind two molecules of fatty acid per molecule protein and more recently, bovine L-FABP was shown to bind two molecules of fatty acid in one binding site [16]. In our experiments, brain FABP appeared to bind one mole of oleic acid and two moles of palmitic acid per mole. The reason for the discrepancy usually observed in the stoichiometry of ligands binding of FABPs is not clear. However, it may be due to several factors such as variable solubilisation or dispersion of fatty acids and partial denaturation, incomplete delipidation or aggregation of FABPs according to the binding assay conditions. However, in view of all available results, FABPs appear to be capable of binding two molecules of fatty acid/molecule.

The sequence of brain FABP presents significant similarities with bovine heart FABP (95%); however, in 17 positions of brain FABP were encountered amino acids not present at similar positions in heart FABP (Fig. 1). Moreover, it is noteworthy that at 20 positions in the sequence, two different

FIGURE 1

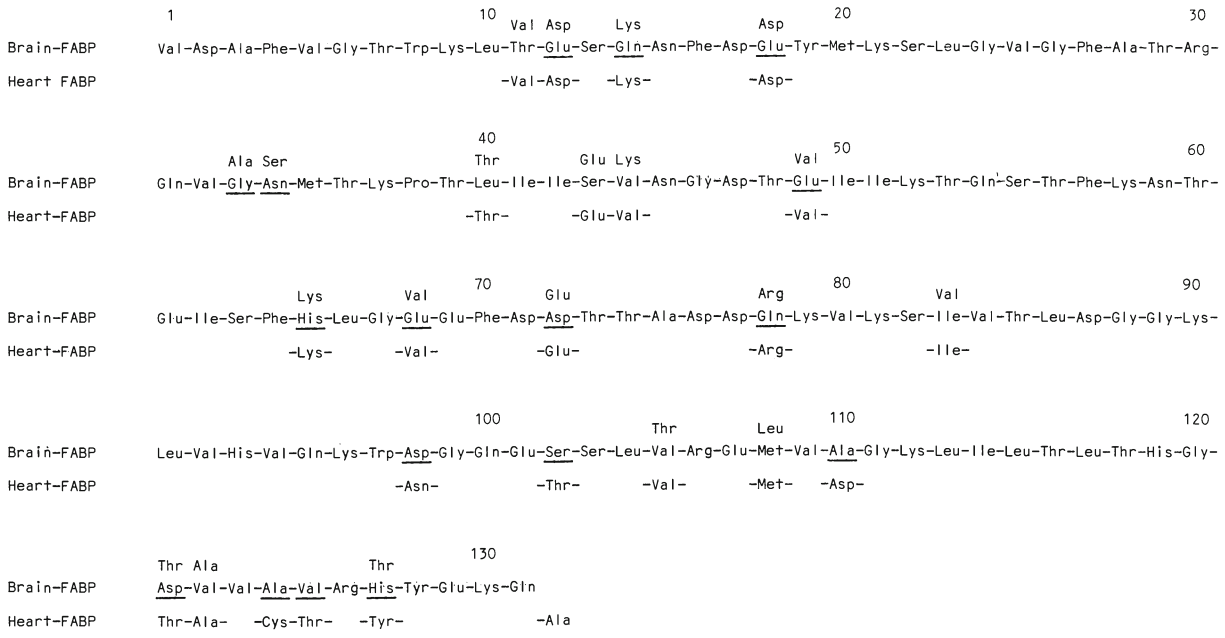

Fig. 1. Sequence of the bovine brain FABP. For 20 positions in the sequence two different amino acids were found. The comparison with the bovine heart FABP [9] is shown and the underlined amino acids correspond to residues not encountered at the same positions in heart FABP.

amino acids were characterized. This result demonstrates that despite of its behaviour in polyacrylamide gel electrophoresis, the isolated brain FABP is microheterogeneous. The observed microheterogeneity reveals the presence of several FABPs in bovine brain. All these brain FABPs are closely related to heart-FABP and, possibly, one of them might be identical to heart-FABP. The occurrence of several FABPs in different tissues has already been observed by others. Indeed, two isoforms of H-FABP that could not be distinguished by polyclonal antisera were described in bovine heart [17]. In the rat mammary tissue, two FABPs were found to be similar to the FABP isolated from rat heart based on the electrophoretic mobilities and amino acid composition [18]. The bovine mammary-derived growth inhibitor was described to have extensive sequence homology to H-FABP and was shown to present two different amino acids in several positions of its primary structure [19].

H-FABP has been proposed to be implicated in the control of energy production in the heart by carrying long-chain fatty acids to mitochondria for β-oxidation [20]. However, the brain oxidizes fatty acids poorly or not at all. Thus, the presence in brain of several FABPs support the suggestion that the different isoforms of FABPs could be implicated in various biological functions.

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Type-specific immunodetection of human heart fatty acid-binding protein with polyclonal anti-peptide antibodies

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Key words: fatty acid-binding protein, synthetic peptide, ELISA, human heart

Summary

In order to develop specific antibodies against human heart cytoplasmic fatty acid-binding protein (H-$FABP_c$), four oligo-peptides of 15–20 amino-acids each and corresponding with different antigenic parts of the human H-$FABP_c$ molecule, were synthesized. Polyclonal antibodies against these synthetic peptides were raised in mice (Balb/C) and rabbits (Flemish giant). When tested in enzyme linked immunosorbent assays (ELISA, antibody-capture assay), antisera against three of the four peptides showed a high immunoreactivity with the synthetic peptide selected for immunization as well as with the native human H-$FABP_c$. Some cross-reactivity with the other synthetic peptides was observed for the rabbit antisera but not for those from mice. Polyclonal antibodies against synthetic peptides can be applied for the specific detection of the native protein in biological preparations containing proteins that show a high degree of homology with the protein to be assayed.

Introduction

Fatty acids play an important role in various aspects of cellular metabolism. Cytoplasmic fatty acid-binding protein ($FABP_c$) is probably involved in the uptake, transport and metabolism of fatty acids, but specific roles remain to be determined. The FABPs are a group of 14–15 kDa proteins, abundantly present (3–5% of protein on weight basis) in the cytoplasm of various mammalian cell types, and capable of binding long-chain fatty acids, fatty acyl-CoA and acyl-L-carnitines (for review see 1). The three main types of FABP identified at present are heart (H-), liver (L-), and intestinal (I-)$FABP_c$, each named after the tissue they were first found in, but in which they are not exclusively expressed [1, 2]. These FABP types show considerable differences in amino acid composition and primary structure (less than 30% sequence homology), resulting in a mutual crossreactivity of polyclonal antisera raised against these FABP types of only 0–5% [3, 4].

However, other FABP types, recently detected in adipose tissue [5], kidney [6] and brain [7] show a high degree of sequence homology, both mutually and with other types. These common sequences can comprise of one or more immunodominant epitopes. Hence, when the $FABP_c$ molecule is presented to the immune system as antigen, this can result in production of antibodies preferably directed against these immunodominant sites. As a consequence of this immunodominance [8] polyclonal antibodies raised against specific types of $FABP_c$ are likely to show crossreactivity with other types.

Therefore, the type-specific immunochemical determination of FABPs in a mixture with other types is hampered. However, the problem of immunodominancy may be circumvented by presenting a small, type-specific part of the $FABP_c$ molecule to the immune system, provided that this part bears appreciable antigenicity [9].

In order to develop type-specific antibodies to human H-$FABP_c$ an antigenic index [10, 11] was computed to identify highly antigenic regions on the human H-$FABP_c$ molecule. Characteristic parts within these antigenic regions were synthesized and conjugates of synthetic peptide and a carrier protein (Keyhole Limpet Hemocyanin) were used for inoculation of rabbits and mice. Immunoreactivity and immunospecificity of the raised polyclonal antisera were tested in ELISA as well as on tissue sections.

The results show that the selected synthetic peptides all were immunogenic as well as antigenic, rendering antisera with a high titer and variable specificity. For those cases in which human H-$FABP_c$ was recognized by the antibodies, the immunoreaction with the native protein was detected at even higher serum dilutions than with the synthetic peptide.

Materials and methods

Peptide selection

Based on the published amino acid sequence of human H-$FABP_c$ [12], putative antigenic sequences were predicted using algorithms for: hydrophilicity [13, 14], α-helix and β-turns [15, 16], surface probability [17, 18], and flexibility [19], culminating in an antigenic index according to Jameson & Wolf [10] and Wolf *et al.* [11]. Strongly homologous regions were avoided. This led to the synthesis of four peptides, designated SP1 (residues 10–25, KLVDSKNFDDYMKSLG), SP2 (residues 37–53, TKPTTIIEKNGDILTLK), SP3 (residues 97–112, QKWDGQETTKVRELID), and SP4 (residues 120–134, THGTAVSTRTYEKEA). Selected sequences showed no significant degree of homology with human L-$FABP_c$, human serum albumin, transferrin, and the constant regions of IgA1, IgM, and IgG subclasses.

Synthetic peptides

Peptides were produced by solid-phase synthesis essentially according to Merrifield [20] modified in detail as described by Van Laar *et al.* [21]. Briefly, Rapid-Amide beads were used essentially using F-moc protected amino acids (Dupont, Wilmington, USA) in the procedure as described for the RaMPS System (Dupont Medical products, Biotechnology Systems, USA). Piperidine was used for deprotection. Elongation was controlled at each step [22], and, in case of a proline, a modification was carried out according to the method described by Kaiser [23]. Final deprotection and cleavage were performed using a mixture of trifluoroacetic acid, phenol and ethanediol followed by precipitation from diethylether. Cysteines with a tertiary butyl protection group were deprotected using mercury(II)acetate.

Peptides were purified as described elsewhere [24]. Briefly, peptides were reduced using β-mercaptoethanol and subsequently purified over G-15 Sephadex in 5% acetic acid. Fractions showing a single peak on reverse phase chromatography in a gradient of 0.1% trifluoroacetic acid in acetonitrile were pooled. Peptides SP1 and SP2 were conjugated to Keyhole Limpet Hemocyanine (KLH) with maleimidobenzoyl-N-hydroxysuccinimide [25] using the cysteine-SH groups as described elsewhere [7]. The peptides SP3 and SP4 were coupled to KLH using a glutaraldehyde coupling method [26]. Control conjugates of the peptides with bovine serum albumin were produced using a carbodiimide coupling [27].

Isolation and purification of $FABP_c$

Human H-$FABP_c$ was isolated from tissue obtained within 12 hours post mortem. Isolation and purification procedures were essentialy as described by Glatz *et al.* [28]. In short, homogenates of human heart tissue (25%, w/v) were prepared in

buffer, consisting of 10 mM Tris-HCl (pH 8.0), 150 mM KCl and 1 mM dithiothreitol. After centrifugation at 2,600 × g for 10 minutes and 105,000 × g for 90 minutes, the final supernatant containing the cytosolic proteins, was concentrated by ultra-filtration using a Diaflo YM5 membrane, and applied to a Sephacryl S200-SF column equilibrated with homogenization buffer. Elution of proteins was monitored spectrophotometrically at 280 nm. FABP containing fractions, identified by Lipidex 1000 assay [28], were pooled, concentrated, dialysed overnight at 4° C against 5 mM Tris-HCl (pH 8.0), and then applied to a Sepharose-Q fast flow column equilibrated with dialysis-buffer. $FABP_c$ was collected after stepwise gradient-elution (0–30 mM NaCl in 5 mM Tris-HCL, pH 8.0). Purity of $FABP_c$ was assessed by SDS-PAGE and isoelectric focusing. Finally, the protein was dialysed against 10 mM K-phosphate (pH 7.4), containing 150 mM NaCl, and stored in aliquots at − 20° C.

Preparation of antibodies

For each peptide two rabbits (Flemish giant) were immunized and boosted 4 weeks later with KLH-SP conjugate in a mixture of PBS and Freund's Complete Adjuvant (FCA). In addition, two rabbits were immunized and boosted with human H-$FABP_c$. Boosting was repeated in week 8 (second boosting) with Freund's Incomplete Adjuvant (FIA) instead of FCA.

Four groups of four female mice (Balb/C) were immunized intra-peritoneally with antigen (human H-$FABP_c$ or KLH-SP conjugates) in an PBS/oil-emulsion [29]. The same emulsion was used four and eight weeks later for the first and the second booster, respectively. The results discussed in this paper were obtained with the sera collected from rabbits and mice two weeks after the second booster.

Enzyme linked immunosorbent assay (ELISA)

Rabbit sera. Antigen (human H-$FABP_c$ or SP1–4) was coated on 96-well polyvinylchloride microtiter plates by overnight incubation at 4° C in 0.1 M carbonate buffer, pH 9.6. Subsequent washing and blocking was carried out with PBS, supplemented with 0.05% (v/v) Tween-20 and 0.1% (w/v) bovine serum albumin. Serially diluted polyclonal antisera were incubated in the microtiter plates for 90 minutes at 20° C. The plates were then washed and goat-anti-rabbit IgG(H + L), linked to horseradish peroxidase, was added. Following 90 min incubation at 20° C the plates were washed again and developed with substrate-solution, containing 0.04% (w/v) o-phenylene diamine and 0.0015% (v/v) H_2O_2. Development was stopped with 4N H_2SO_4, and extinction was read at 492 nm using a MkII Plus microplate reader (Flow Lab).

Mouse sera. Similar procedures as for rabbit sera were followed, except for the blocking and washing steps, which were carried out with PBS, supplemented with 0.1% (w/v) gelatin and with PBS, supplemented with 0.1% (w/v) gelatin and 0.05% (v/v) Tween-20, respectively. Bound antibodies were detected with goat-anti-mouse IgG, linked to alkaline phosphatase, and subsequent development was carried out with substrate-solution, containing 0.1% (w/v) p-nitrophenyl phosphate in 0.01 M diethanol-amine and 0.01 M $MgCl_2$, pH 9.8. Extinction was read at 405 nm.

Immunohistochemistry

Frozen as well as paraffin embedded tissue sections (4 μm) of human heart muscle, psoas muscle, liver, and duodenum (frozen tissue only) were used. Prior to embedding in paraffin, tissues were fixed by submersion in buffered formalin, pH 7.0 (4%, v/v), for at least 24 hours. Frozen tissue sections were fixed by exposure (10 min) to paraformaldehyde-vapour in a dessicator under light vacuum, or by submersion in cold (− 20° C) acetone for 20 min. Staining results with either fixation method showed no appreciable differences.

All antisera were used in a 1 : 500 dilution in PBS, supplemented with 0.1% (w/v) bovine serum albumin. Bound rabbit- and mice-antibodies were

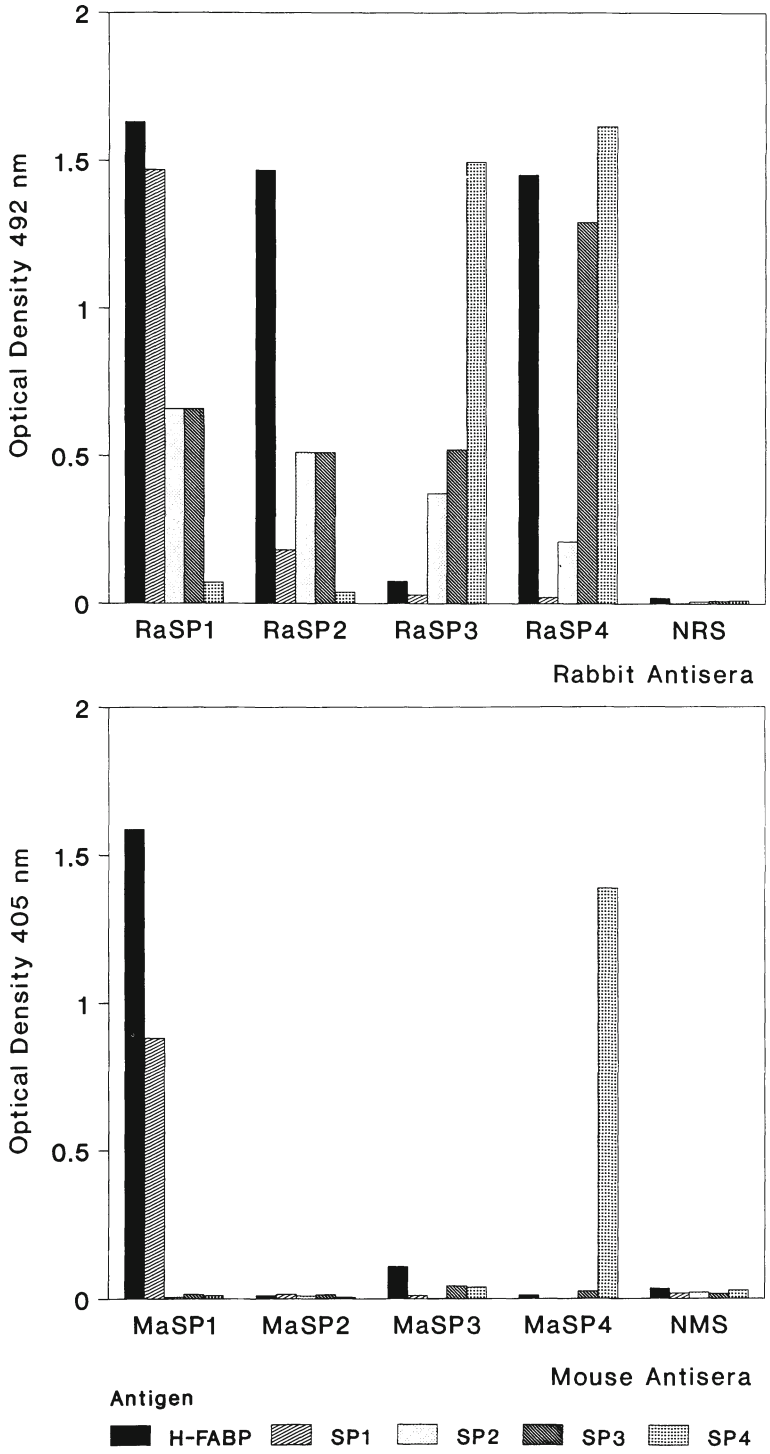

Fig. 1. Specificity of polyclonal rabbit (upper panel) and mice (lower panel) antisera raised against the synthetic peptides SP1 to SP4 (RaSP's and MaSP's, respectivily) tested in ELISA for their reaction with the native protein (human H-FABP$_c$), and with the four synthetic peptides. Results are shown of typical experiments with sera from one rabbit per peptide and of mean values obtained with sera of four mice per peptide (differences among individual mice were less than 10%). Normal rabbit serum (NRS) and normal mouse serum (NMS) were used as negative controls. Serum dilution was 1 : 1000 (rabbits) or 1 : 400 (mice).

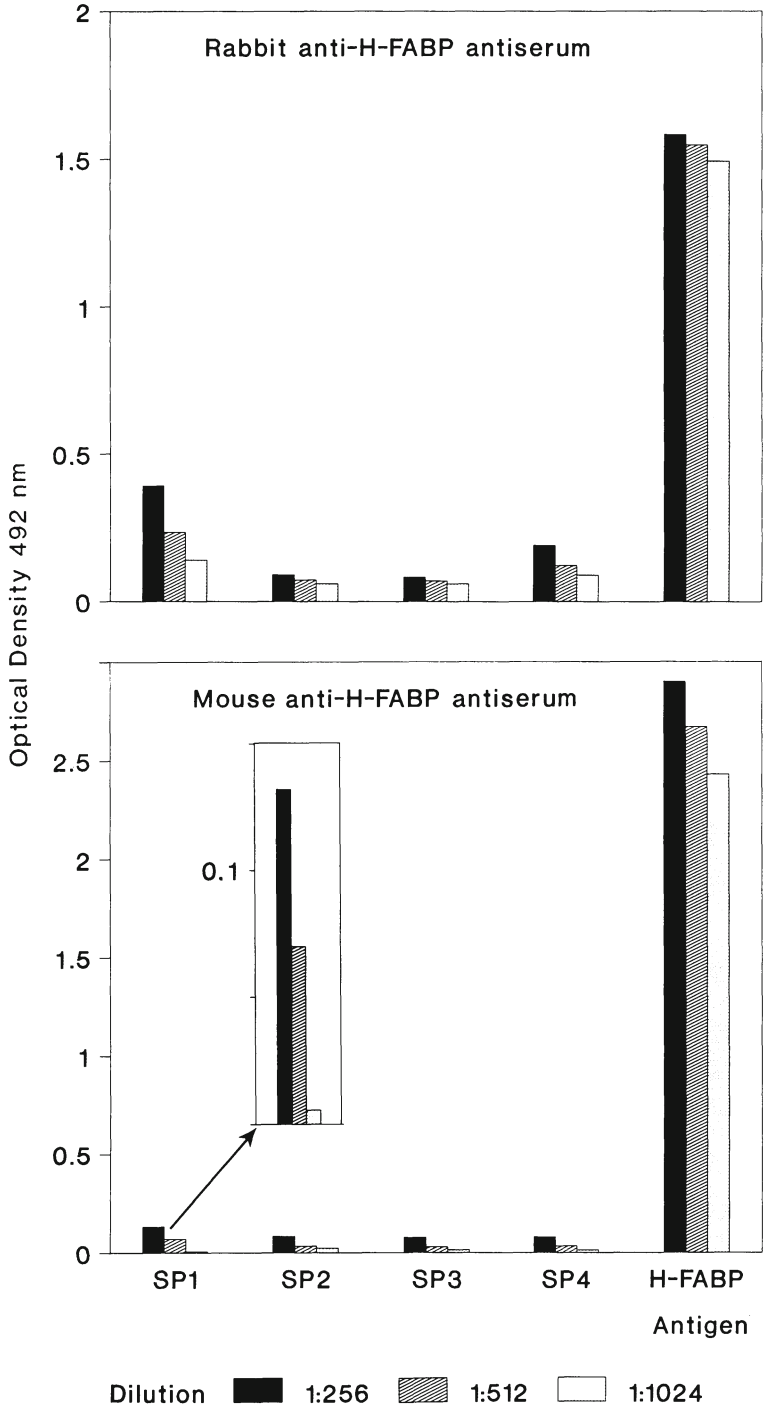

Fig. 2. Immunoreactivity, at different dilutions, of polyclonal rabbit (upper panel) or mice (lower panel) anti-human H-FABP$_c$ antisera tested in ELISA on human H-FABP$_c$ and the synthetic peptides SP1 to SP4. Results are shown of typical experiments with serum from one rabbit and of mean values obtained with sera of three mice (differences among individual mice were less than 10%). Immunoreaction of mouse anti-H-FABP$_c$ antiserum with SP1 (enlarged in insert of lower panel) is representative for the reaction of this anti-serum with the other SP's.

detected with goat-anti-rabbit IgG and rabbit-anti-mouse Ig, respectively, conjugated with horseradish peroxidase. Diaminobenzidine was used as substrate. Tissue sections were counterstained with haematoxylin.

Results

Analysis of anti-peptide antibody response in ELISA

In all rabbits inoculation and subsequent boosting with KLH-SP-conjugates evoked an immunoresponse, rendering anti-SP antisera (RaSP) that reacted strongly with the specific synthetic peptide (Fig. 1). Antisera against three of the four peptides could also recognize the native protein, human H-FABP$_c$. Moreover peptide antiserum RaSP2 reacted even stronger with human H-FABP$_c$ than with its immunogen. RaSP3 and RaSP4 showed a strong crossreaction with their mutual antigens. However, the reaction of RaSP3 with human H-FABP$_c$ is negligible. Surprisingly, rabbit antisera raised against purified human H-FABP$_c$ (RaH-FABP), showed no or hardly any crossreactivity with any of the free synthetic peptides (a typical example is shown in Fig. 2).

In mice only two of the four anti-SP antisera (MaSP) showed anti-SP-specific immunoreaction when tested in ELISA (MaSP1 and MaSP4 in Fig. 1). Only one of these (MaSP1) also showed a reaction with the native protein (Fig. 1). Polyclonal antisera raised against purified human H-FABP$_c$ in mice (MaHFABP) showed high antibody titers against the native protein, but no immunoreactivity with the synthetic peptides (Fig. 2). Control sera, i.e. normal rabbit and mice sera, showed no immunoreaction (Fig. 1).

Immunohistochemical studies

Crossreactivity of the raised antisera with other FABP types was studied immunohistochemically on frozen and paraffin sections of various human tissues. With rabbit antisera the results on paraffin sections were comparable to those on frozen sections of the same tissues. Cytoplasmic staining of myocardial myocytes was observed with the rabbit antisera RaHFABP, RaSP1, and RaSP2. M. psoas and m. mucosae myocytes, and hepatocytes remained unstained when the latter three antisera were used. Antisera RaSP3 and RaSP4 did not stain any of the cell types studied, including cardiomyocytes.

Mouse sera, even at antiserum dilutions as low as 1 : 100, were not able to stain (frozen or paraffin) sections of human myocardium, liver, psoas muscle, or duodenum.

Discussion

Peptides representing unique amino acid sequences which have a high score on the antigenic index were used as specific antigenic determinants for the human heart cytoplasmic fatty acid-binding protein. Four peptides were selected for synthesis on the basis of a Jameson & Wolf prediction [10]. The synthetic peptides were used for the generation of polyclonal antisera in rabbits and mice. In rabbits each of the selected peptides proved to be immunogenic and antigenic. Three out of four specific polyclonal rabbit antisera also recognized the native protein (H-FABP$_c$). In mice only two out of four peptides were both immunogenic and antigenic. The specific polyclonal antiserum raised against one of these also recognized H-FABP$_c$. Immunohistochemistry showed that only the cytoplasm of cardiomyocytes was stained, and that only two of the four specific polyclonal rabbit antisera were able to do so. None of the specific polyclonal mice antisera were able to stain any of the studied cell types.

The antibodies of one of the rabbit antisera, RaSP3, did recognize the free oligopeptide (SP3) but not its equivalent in the native protein, either under condition of ELISA or in tissue sections of human myocardium. This might indicate that there is a conformational difference between the free oligopeptide SP3 and the sequence of this peptide in the native protein.

Taken together, the results of the ELISA and the

immunohistochemical experiments suggest that the synthetic peptides SP1 and SP2, and their equivalents in the native FABP molecule, are stable and easily recognized structures. Antibodies in the RaSP4 antiserum recognize the native protein in ELISA but, under the present conditions, not in tissue sections of human myocardium. The latter may have been caused by slight denaturation of human H-FABP$_c$ during fixation of the tissues preceding the immunohistochemistry. The use of milder fixatives for immunohistochemistry is under consideration, although this could lead to elution of cytoplasmic FABP during preparation of the tissues. As expected, none of the studied antisera crossreacted with proteins in tissue sections of human liver.

Only two groups of mice generated antisera which reacted with the immunizing synthetic peptide and, of these, MaSP1 also reacted with the native protein. The polyclonal antiserum raised against SP4 only reacted with the free oligopeptide SP4. Of the tested synthetic peptides only SP1 and SP4 seem to be immunogenic in the immune system of mice and antigenic for the generated antibodies. SP2 and SP3 do not seem to be immunogenic in the immune system of mice, in the form of a KLH-SP conjugate, or to be antigenic at all, in the form of a free oligopeptide.

The finding that polyclonal rabbit and mice antisera, raised against purified human H-FABP$_c$, show no immunoreactivity in ELISA with any of the synthetic peptides suggests that at least one region on the human H-FABP$_c$ molecule is more immunogenic that the regions defined by the amino acid sequences of the used synthetic peptides. Apparently, this unidentified immunogenic region was not distilled from the Jameson & Wolf [10] prediction data. The latter can be explained by the fact that the Jameson & Wolf approach predicts antigenicity on the basis of the amino acid sequence of the peptide and the properties of its individual amino acids, thus predicting only lineair determinants. Structural determinants or discontinuous epitopes, e.g. immunogenic and antigenic sites that bridge a gap or a turn in the amino acid chain, cannot be predicted using this approach. Because the synthetic oligopeptides used in our experiments mutually have only two pairs of adjacent amino acids in common at the most, crossreactivity of an antiserum raised against one of the used synthetic oligopeptides with one of the other selected oligopeptides (cf. RaSP3 and RaSP4) may indicate that antibodies not only recognize specific amino acid sequences but also certain tertiairy structures in a protein [30]. On the other hand, polyclonal sera contain various antibodies which will have a broad affinity range for recognition of the peptides and the native protein. Furthermore, some of the antibodies may recognize one peptide whereas the other peptide is recognized by different antibodies.

It is expected that the polyclonal rabbit antisera raised against the peptides SP1, SP2, SP3, or SP4, preferably in combination with a monoclonal antibody directed against an antigenic site of the native protein not recognized by the polyclonal antisera, can be used for detection of the native protein in biological preparations by means of a capture-ELISA.

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Bifunctional lipid-transfer: fatty acid-binding proteins in plants

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Key words: lipid, transfer, protein, fatty acid, binding, plants

Summary

A cytosolic protein, able to facilitate intermembrane movements of phospholipids *in vitro,* has been purified to homogeneity from sunflower seedlings. This protein, which has the properties of a lipid-transfer protein (LTP), is also able to bind oleoyl-CoA, as shown by FPLC chromatography. This finding, in addition to previous observations suggesting that a lipid-transfer protein from spinach leaves can bind oleic acid and that oat seedlings contain a fatty acid-binding protein with similar features than lipid transfer proteins, provides a clear demonstration that plant cells contain bifunctional fatty acid/lipid transfer proteins. These proteins can play an active role in fatty acid metabolism which involves movements of oleyl-CoA between intracellular membranes.

Abbreviations: FABP – Fatty Acid-Binding Proteins, LTP – Lipid-Transfer Protein, PC – Phosphatidylcholine, PI – Phosphatidylinositol, PE – Phosphatidylethanolamine, pI – Isoelectric point

Introduction

Plant cells contain proteins able to facilitate in vitro intermembrane movements of phospholipids [1]. These proteins, called lipid transfer proteins (LTP), have been purified to homogeneity from various plant tissues like spinach leaves [2], maize [3] or castor bean [4] seedlings. Plant LTP have similar properties, like molecular mass (about 9 kDa), basic pI (around 9) and a broad non specificity for phospholipids since phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) are transferred from liposomes (donor membranes) and mitochondria (receptor membranes). These plant LTP have properties roughly similar to those of nonspecific phospholipid transfer proteins from animal tissues like bovine liver which are able to transfer phospholipids as well as sterols [5, 6]. In addition to these LTPs, proteins able to bind oleoyl-CoA and oleic acid (fatty acid-binding proteins; FABP) have been isolated from oat seedlings [7]. The experiments were based on the incubation of the protein with the fatty acids and then a separation either by the dextrancoated charcoal method or by isoelectric focusing. The fact that this protein has similar properties than plant LTPs led to study the eventual fatty acid binding by spinach LTP. The isoelectric focusing technique indicated that a binding of oleic acid indeed occurs on spinach LTP [8].

In order to provide decisive arguments in favour of this double property of transferring and binding lipids, it was tempting to study oil-forming seeds, like those of sunflower, which have intense fatty acid metabolism linked to the synthesis of triacylglycerols. Starting from germinating sunflower seeds, a pure LTP was isolated, having properties roughly similar to those of other plant LTP. When assayed with oleoyl-CoA, a binding to the sunflower protein was observed as indicated by FPLC chro-

matography of the fatty acid/protein complex. The present work demonstrates that bifunctional lipid transfer/fatty acid binding proteins are present in sunflower seedlings. At the light of these results and the previous observations on LTP from spinach and FABP from oat, it is concluded that plant cells contain bifunctional fatty acid-binding/lipid-transfer proteins.

Materials and methods

Materials

Sunflower seeds (*Helianthus annuus* L.c.v. rodeo) were obtained either from experimental farms of CETIOM (Paris). Cholesteryl (1–14C) oleate was purchased from Amersham. ^{3}H phosphatidylcholine was prepared as previously indicated [9].

Preparation of the protein extracts

Sunflower seeds (400 g) were grown on filter paper at 30° C in the dark for four days. All the following manipulations were carried out at 4° C. Seedlings were ground in 500 ml of 0.1 M Tris-HCl (pH 7.5), 0.4 M sucrose, 6 mM Na4 EDTA, 1 mM cysteine, 9 mM β-mercaptoethanol. After readjusting the pH to 7.5, the homogenate was filtered through a double layer of cheesecloth and centrifuged at 8,000 *g* for 30 min. After lowering its pH to 5.1, the supernatant was centrifuged. The pH of the supernatant was then readjusted to 7.5 and proteins were allowed to precipitate by adding 52 g of (NH4)2 SO4 per 100 ml. The mixture was stirred overnight and then centrifuged at 8,000 *g* for 30 min. The pellets were resuspended in a minimal volume of 10 mM sodium phosphate (pH 7.2), 10% glycerol, 8 mM β-mercaptoethanol, 3 mM NaN3 and the resulting suspension was dialyzed for 5 hours against 20 volumes of the same buffer in order to eliminate (NH4)2 SO4. After lowering the pH to 5.1, the dialyzate was centrifuged at 8,000 *g* for 30 min to remove insoluble material. The supernatant, after pH adjustment at 7.2, was used as the protein extract for further purifications.

Transfer assay

Transfer assays were carried out as previously described [2]: protein extracts were incubated with 3PC (transferable lipid) and 14C cholesteryl oleate (non transferable lipid) liposomes and with purified maize mitochondria at 30° C for 30 min. The mitochondrial pellets were recovered by centrifugation, resuspended in 2% Triton X-100 and the radioactivity measured in a liquid scintillation counter. The transfer activity of the protein extract was expressed as nmol of PC transferred to mitochondria.

Binding assay

The protein fractions were incubated with 100 nmol of oleoyl-CoA (specific radioactivity: 2 GBq · $mmol^{-1}$; Amersham) for 30 min at + 4° C. The mixture was then chromatographed on a FPLC MonoS column (Pharmacia).

Electrophoresis

Protein extracts were analyzed by acrylamide-SDS gel electrophoresis using gel slabs made from a 7.5–15% acrylamide gardient. Gels were coloured using either Coomassie blue or AgNO3 [2, 3].

Results

Purification of the sunflower protein

The operations used to purify the protein are summarized in Table 1.

Step 1. The protein extract obtained after ammonium sulphate precipitation is able to transfer phosphatidylcholine from liposomes to mitochondria with a specific activity of 0.08 nmol mg $protein^{-1}$ min^{-1}.

Step 2. The protein extract was loaded on a Sephadex G75 column equilibrated with 10 mM sodium

phosphate (pH 7.2), 10% glycerol, 3 mM sodium azide and 8 mM β-mercaptoethanol (Buffer A). The active fractions (78–100) were pooled; they correspond to low molecular-mass proteins (Fig. 1A).

Step 3. The active fractions were applied to a DEAE-Trisacryl column equilibrated with buffer A in order to remove the acidic fractions which have a negligible activity. The effluent was directly passed through a CM-Sepharose column equilibrated with the same buffer. Bound proteins were eluted using a linear 0.01–0.25 M phosphate gradient in buffer A. Two majors peaks of activity were observed, CM1 and CM2, which correspond to a salt concentration respectively of 160 and 200 mM (Fig. 1B). The specific activity of the CM2 peak is equal to 2.88 nmol mg^{-1} min^{-1}; a similar value was found for the CM1 peak (data not shown).

Step 4. The purification of the protein was achieved by FPLC. CM2 fraction was chromatographed on a MonoS column (Pharmacia) equilibrated with Buffer B (Buffer A minus glycerol) (Fig. 1C). Proteins were eluted with a 0–0.5 M NaCl gradient in the same buffer. The chromatogram obtained from CM2 showed a major peak corresponding to a salt concentration of 0.23 M. Fractions 22 and 23 were pooled and used as a purified protein having a specific activity of 10.56 nmol mg^{-1} min^{-1}. A 132-fold purification was noted.

Properties of the sunflower protein

The analysis by SDS-PAGE of CM2 fraction showed a single band with an apparent molecular mass of 9 kDa. The same band was detected in the CM1 peak, but with other additional bands; for this reason, further purifications were made on only the CM2 peak. The value of 9 kDa is in good agreement with those obtained from the other plant LTP [1], but it differs from animal nonspecific proteins which have higher molecular masses [5, 6]. It is to be noted that this protein is detected as a distinct band in electrophoregrams of the total extract suggesting that the protein is abundant in sunflower seedlings. This can be correlated to the fact that maize LTP represents 2 to 4% of the total soluble proteins as determined by immunochemical methods [10, 11].

The observation that sunflower protein is bound on CM Sepharose indicates a high pI. This was confirmed by a chromatofocusing technique which indicated a pI near 9 (data not shown). This is in agreement with the high pI found in the case of maize, spinach and castor bean proteins although some acidic proteins, but less abundant, have been detected in these plants [1].

Interestingly, the transfer activity is associated with a double peak of activity (CM1 and CM2). The existence of several peaks of activity in separations on CM column has also been showed during the isolation of maize and spinach LTP. That suggests the existence of different isoforms, which could have the same molecular mass and slightly different pI.

Table 1. Purification procedure of sunflower protein. The different steps of purification of lipid transfer protein from sunflower seedlings is described in Materials and methods. The various steps correspond respectively to the protein extract after dialysis (step 1), to the pooled active fractions from Sephadex G75 column (step 2), the active fraction from CM-Sepharose column (isoform CM2) (step 3) and the final pure protein from the MonoS FPLC column (step 4). The specific activity is expressed as nmol of phosphatidylcholine transferred from liposomes to mitochondria per mg of protein per min.

Steps	Protein mg · ml-1	Volume ml	Total Protein mg	Specific activity nmol · mg^{-1} · min^{-1}	Purification
Crude extract (step 1)	44	120	5280	0.08	1
Sephadex G75 (step 2)	1.5	315	472	1.06	13.25
CM2 (step 3)	0.36	149	53.6	2.88	37
MonoS (step 4)	0.32	4	1.3	10.56	132

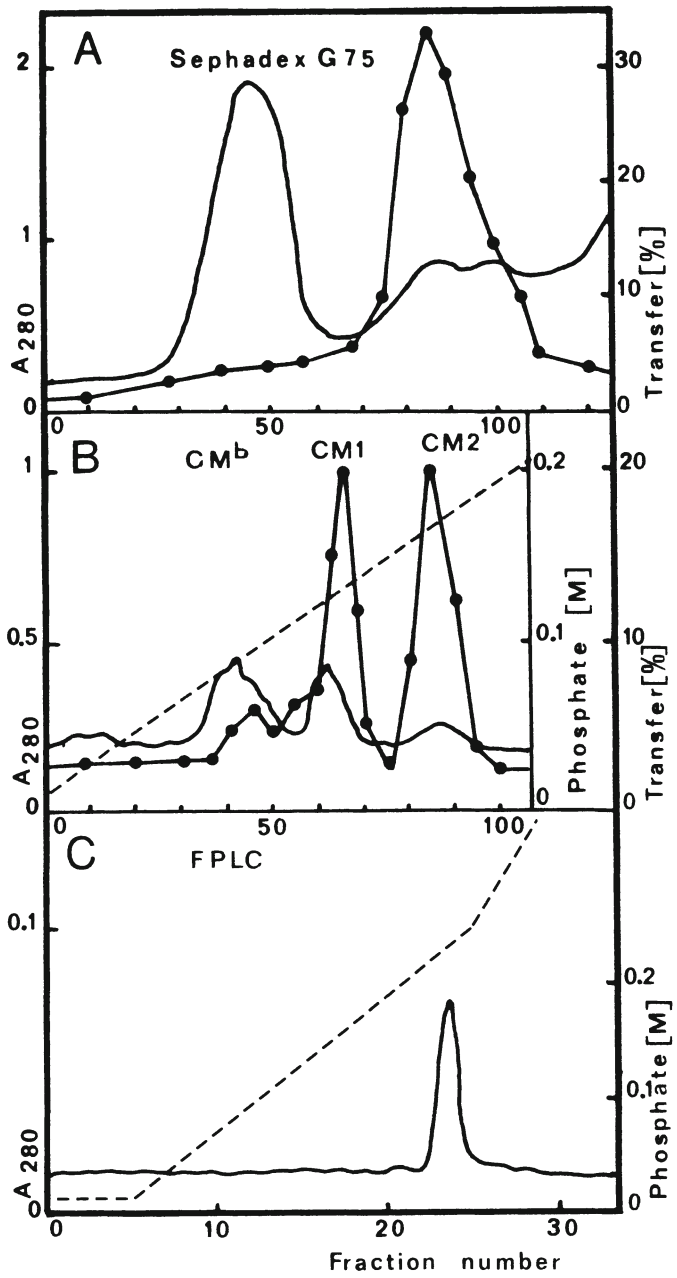

Fig. 1. Elution pattern of sunflower proteins. A: Chromatography on Sephadex G-75. The protein extract (5 g) was loaded on a 5 × 70 cm column equilibrated with 10 mM sodium phosphate (pH 7.2), 10% glycerol, 3 mM sodium azide and 8 mM β-mercaptoethanol. The flow rate was 80 ml h-1 and 15 ml fractions were collected. The absorbance was measured at 280 nm and the transfer activity was expressed as a percentage of phosphatidylcholine transfered from liposomes to mitochondria using 3 ml of each fraction. Fractions 78–100 were pooled. B: CM-Sepharose. The unbound effluent from DEAE-Trisacryl (step 3) were applied to a 2.6 × 25 cm column equilibrated with the same buffer indicated in A. Elution was effected using a linear gradient formed by 11 of the equilibration buffer and 11 of the same one made up to 250 mM sodium phosphate. The flow rate was 60 ml h-1 and 25 ml fractions were collected. The salt concentration was determined by measuring their conductivity. Absorbance at 280 nm and transfer activity are indicated as in A. Fractions 65–85 (CM1) and 86–95 (CM2) were pooled separately. C: FPLC on MonoS. 1.3 mg of the CM2 fraction were applied to a MonoS column (Pharmacia) equilibrated with 10 mM sodium phosphate (pH 7.2), 3 mM sodium azide, 8 mM β-mercaptoethanol. Proteins were eluted using a 0.5 M NaCl gradient prepared in the same buffer. The flow rate was 60 ml h-1 and 1 ml fractions were collected. Absorbance was monitored at 280 nm. The shape of the gradient is indicated by a discontinuous line. Fractions 22 and 23 were pooled.

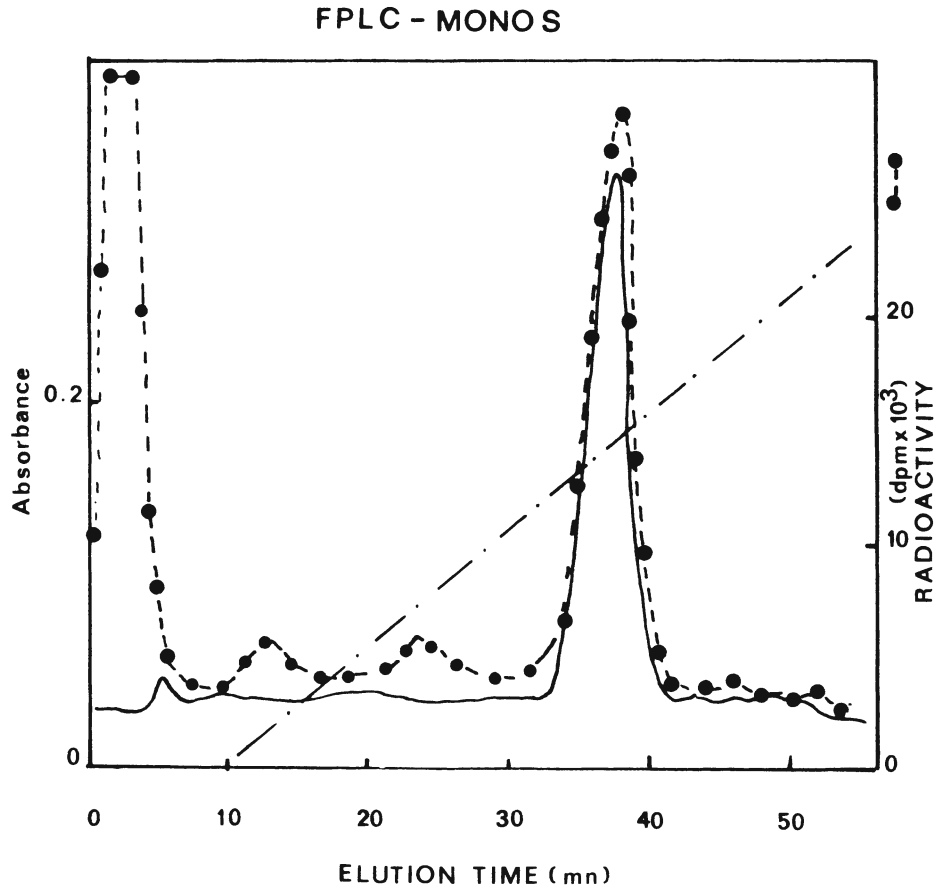

Fig. 2. Binding of oleoyl-CoA on the sunflower protein. After incubation of 0.9 mg of CM2 fraction with 100 nmol of 14C-oleoyl-CoA (see Materials and methods), the mixture was separated by chromatography on a MonoS column in the experimental conditions indicated in Fig. 1C. The radioactivity (circles) is expressed as dpm per ml of fraction.

Binding of oleoyl-CoA

When the CM2 fraction was incubated with oleoyl-CoA, a radioactive peak, corresponding to the protein, was detected after separation of the incubation mixture by FPLC on a Mono S column (Fig. 2). Around 40% of the total 14C label was recovered in the protein peak.

NH2-terminal sequence

A NH2-terminal sequence comprising 24 residues has been determined for the CM2 fraction (Fig. 3). The most important feature of this sequence is its hydrophobic character, especially between residues 6–17. The property of this part of sequence seems to be common to other plant LTP (maize, spinach or castor bean) [12].

Discussion and conclusions

The present work establishes that a protein highly purified from an oil-forming seed, is not only able to transfer phospholipids between membranes but also to bind oleoyl-CoA. We propose that this protein belongs to a category of bifunctional proteins having the ability to bind and transfer lipids.

The sunflower protein, isolated in this work, shares with plant LTPs common properties: same molecular mass and around 9 kDa and high pI.

			*			*			10		
Sunflower		Ile	Thr	Cys	Asn	Asp	Val	Thr	Gly	Asn	Leu	Thr
Maize	Ala	Ile	Ser	Cys	Gly	Gln	Val	Ala	Ser	Ala	Ile	Ala
Spinach	Gly	Ile	Thr	Cys	Gly	Met	Val	Ser	Ser	Lys	Leu	Ala
Castor bean		Val	Asp	Cys	Gly	Gln	Val	Asn	Ser	Ser	Leu	Ala
		*						20				
Sunflower	Pro	Cys	Leu	Pro	Tyr	Leu	Arg	Ser	Ser	Gly	Lys	Pro	Thr
Maize	Pro	Cys	Ile	Ser	Tyr	Ala	Arg	Gly	Gln	Gly	Ser	Gly	Pro
Spinach	Pro	Cys	Ile	Gly	Tyr	Leu	Lys	Gly	Gly	Pro	Leu	Gly	Gly
Castor bean	Ser	Cys	Ile	Pro	Phe	Leu	Thr	Gly	Gly	Val	Ala	Ser	Pro

Fig. 3. NH2-terminal sequence of LTP isolated from sunflower seeds (present work), maize seeds [12], spinach leaves [12] and castor bean seeds [13, 14].

The determination of the NH2-terminal sequence of the sunflower protein confirmed its homology with other plant LTPs. A comparison between those sequences shows a high homology; the percent match is respectively of 29.2%, 33.3% and 20.8% with spinach, castor bean and spinach proteins which have, respectively, in total 91, 92 and 93 residues. Interestingly, the positions of cysteine residues (3 and 13 in sunflower and castor bean; 4 and 14 in maize and spinach) are conserved. The other cysteines, found in a total number of 8, in maize, castor bean and maize, are also located in the same positions in the LTPs [12–15]. It is supposed that these cysteines play an important role in the mode of action of the protein. In contrast, only one cysteine residue was detected in rat liver FABP [16].

The sunflower protein transfers PC and, in addition, like other plant LTP, PI or PE (data not shown). In contrast, animal tissues contain different categories of phospholipid transfer proteins, transferring specifically PC or preferentially PI or transferring a large diversity of phospholipids [5, 6]. These latter proteins, designated as non specific phospholipid transfer proteins, are also able to transfer sterols; for this reason, they were also called sterol-carrier protein 2 [17]. This protein is distinct from sterol-carrier protein 1 which is highly probably identical to FABP [16, 18]. The non specific phospholipid transfer proteins from animal tissues share with plant LTP several properties (low molecular mass (14kDa) and basic character).

The observation that sunflower protein binds oleoyl-CoA leads to the conclusion that this protein belongs to the category of FABP This binding was shown by a separation of oleoyl-CoA and protein by a cationic column since this protein is basic. In preliminary experiments carried with sunflower protein, other assays were carried out and confirmed this binding: gel filtration on Sephadex G75 and Lipidex assay [16]. From the experiments of Fig. 2, it can be calculated that 0.4 mol of oleoyl-CoA can be bound to 1 mol of sunflower protein since 40% of the ligand (given at the amount of 100 nmol) are recovered bound to 100 nmol of protein. This suggests that sunflower protein has one site of binding of fatty acids per mole. The stoichiometry of fatty acid binding to animal FABPs has been reported to vary from one (a value largely found for several FABP) to two [16, 18].

Interestingly, a protein with a molecular mass of 8.7 kDa, able to bind fatty acids, has been purified from oat seedlings [7]. This protein has a pI of either of 8.4 (when the protein is delipidated) or 4.8 (with the lipidated protein). In these experiments, the binding assay was based on the isolation of protein-fatty acid complex by isoelectric focusing. The same assay was carried out with spinach LTP, indicating that this protein has the ability to bind fatty acids [8].

The properties of plant FABP-like proteins are different from those of animal FABP. The molecular mass of these latter proteins vary from 12 to 14 kDa while their pI vary from 4.3 to 9.3, the rat liver FABP exhibiting a pI around 5.2 to 6.9 [16, 18]. Isoforms of animal FABP have been found, as for plant FABP. Animal FABP, like plant FABP/LTP, are abundant proteins, accounting for 4 to 5%

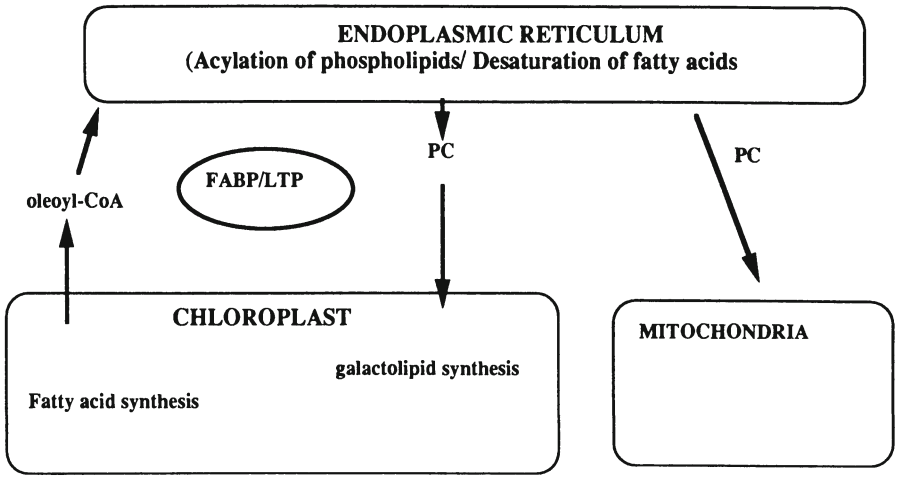

Fig. 4. Scheme indicating the cooperation between organelles (endoplasmic reticulum and chloroplast) for the biosynthesis of galactolipids in plant cells.

of cytosolic proteins. Based on SDS-PAGE of crude extracts, sunflower protein seems to be abundant. A precise quantitation has been made by ELISA for maize LTP, indicating that this protein represents 2 to 4% of the total protein extract. If it is assumed that all plant LTP have a fatty acid binding ability, it can be concluded that, as animal FABP, plant FABP are abundant in the cytosol.

One major question about plant FABP concerns their *in vivo* role. As for animal cells, it can be assumed that plant FABP i/ facilitate the intracellular transport of fatty acids in the cytosol; ii/ regulate the intracellular pool of fatty acids and acyl CoA esters by storing these compounds since FABP are abundant; iii/ exert a protecting effect by avoiding large concentrations of fatty acids and acyl-CoA esters in the cytosol.

Another possible involvement of FABP, specific for plant cells, could be their participation to lipid metabolism. It is accepted now that the site of fatty acid synthesis in plants is the chloroplast (or the plastid) [19]. The fatty acids synthesized are exported in the form of oleoyl-CoA which is transported to the endoplasmic reticulum and then used for the synthesis of polyunsaturated fatty acids (Fig. 4). The polyunsaturated fatty acids (linoleic or linolenic acids) are then transported to the plastid, esterified to phospholipid molecules, and used for the synthesis of galactoplipids. Alternatively, in oil-forming seeds, phosphatidylcholine can be used as an acyl donor for triacylglycerol synthesis [19]. A dual role for the lipid transporting proteins is thus plausible.

For future investigations, it will be necessary to provide decisive arguments in favour of the *in vivo* role of plant FABP/LTP. One possibility is to follow the fluctuations of the amount of proteins during various physiological processes like germination of seeds or greening of leaves. Another way is to use the site-directed mutagenesis approach to determine precisely the site of interaction of fatty acids. This could be made with maize LTP since a full-length cDNA has been isolated and characterized [12]. The study of the expression of this cDNA in yeast and *E. coli* is thus envisaged. For long-term research, the use of the anti-sense mRNA approach or the preparation of transgenic plants can provide decisive informations about the role of FABP/LTP in plant cells.

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Characteristics of fatty acid-binding proteins and their relation to mammary-derived growth inhibitor

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Key words: fatty acid-binding proteins, mammary-derived growth inhibitor, types, isoforms, subcellular distribution

Summary

Based on sequence relationships the cytoplasmic fatty acid-binding proteins (FABPs) of mammalian origin are divided into at least three distinct types, namely the hepatic-, intestinal- and cardiac-type. Highly conserved sequences of FABPs within the same type correlate with immunological crossreactivities. Isoforms of hepatic-type FABP are found in several mammalian species and for bovine liver FABP specific shifts in isoelectric points upon lipidation with fatty acids are observed. Isoforms of intestinal-type FABP are not known and the occurrence of cardiac-type isoforms so far is confined to bovine heart tissue. A bovine mammary-derived growth inhibitor (MDGI) is 95% homologous to the cardiac-type FABP from bovine heart. Dissociation constants of FABP/fatty acid complexes are in the range of 1 μM and 1 : 1 stoichiometries are usually found, but the neutral isoform of hepatic FABP from bovine liver binds 2 fatty acids. On subcellular levels hepatic- and cardiac-type FABPs are differently distributed. Though mainly cytosolic in either case, immunoelectron microscopy as well as a gelchromatographic immunofluorescence assay demonstrate the association of hepatic FABP in liver cells with microsomal and outer mitochondrial membranes and with nuclei, whereas in heart cells cardiac FABP is confined to mitochondrial matrix and nuclei. In mammary epithelial cells MDGI is associated with neither mitochondria nor endoplasmic reticulum, and is expressed in a strictly developmental-dependent spatial and temporal pattern. The specific role proposed for MDGI is to arrest growth of mammary epithelial cells when they become committed to differentiation in the mammary gland.

Introduction

Physiological significances of fatty acid-binding proteins (FABPs) in intracellular lipid metabolism are discussed since their first description [1]. Nevertheless, functions beyond a mere carrying of fatty acids remain to be substantiated. Sequence analyses reveal fatty acid-binding proteins as members of a family of low molecular weight proteins comprising gastrotropin [2], cellular retinoid binding proteins, myelin P2 protein, aP2 protein from adipose tissue [3] and the mammary-derived growth inhibitor [4]. All these proteins act intracellularly except gastrotropin and mammary-derived growth inhibitor (MDGI), which act extracellularly *in vitro*.

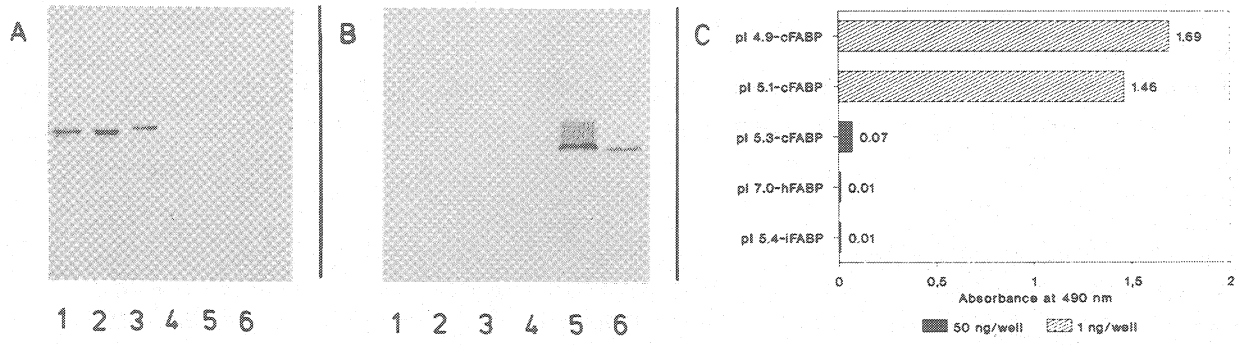

Fig 1. Crossreactivity of antisera with different types of fatty acid-binding proteins. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunostained with affinity purified antibodies against bovine cFABP (panel A) and bovine hFABP (panel B). 1, Bovine pI 4.9-cFABP; 2, bovine pI 5.1-cFABP; 3, human pI 5.3-cFABP; 4, bovine pI 5.4-iFABP; 5, bovine intestinal mucosa cell cytosol; 6, bovine pI 7.0-hFABP. Panel C, intensity of peroxidase staining in ELISA specific for bovine cFABP.

Typology of fatty acid-binding proteins and mammary-derived growth inhibitor

Within the family of fatty acid-binding proteins three different types are unequivocally established up to now and are named by the source of their first isolation. The classification into hepatic-, intestinal- and cardiac-type fatty acid binding protein, abbreviated hFABP, iFABP and cFABP respectively, is based on immunological criteria and reflects a homology of usually ~30% between the different types and 80 to 90% within proteins of the same type. In order to demonstrate this a collection of FABPs was immunostained after SDS-polyacrylamide gelelectrophoresis and subsequent Western blotting (Fig. 1). The use of anti-bovine hFABP-antibodies [5] leads to an immunoreactive band in cytosolic proteins of bovine intestinal mucosa and demonstrates the occurrence of hFABP apart from iFABP in that tissue (Fig. 1B), as previously shown for rat tissue [6]. Pure bovine iFABP does not crossreact with antibodies against hFABP and cFABP. With antiserum against this protein the occurrence of iFABP in enterocytes of bovine small intestine and absence from heart and liver tissue was established [7]. The polyclonal antibodies raised against bovine cFABP additionally crossreact with human cFABP [8], whereas the other types remain unstained (Fig. 1A).

Yet immunological methods with higher stringency, like a sandwich ELISA employing affinity purified antibody for antigen capture and a second one for detection [9], can discriminate between the same FABP-type from different species (Fig. 1C). Even the two isoforms of the cardiac-type fatty acid-binding protein reported for bovine heart cytosol (pI 4.9- and pI 5.1-cFABP) [10] show a graduated response under these conditions. In Fig. 1C the intensity of the peroxidase staining reaction used in the ELISA is shown, i.e. upon application of 1 ng of each isoform, bovine pI 5.1-cFABP exhibits somewhat less than 90% of the immunoreactivity of bovine pI 4.9-cFABP,due to the use of a polyclonal antibody raised against the latter. With this antibody and as much as 50 ng antigen only a weak response is found for human cFABP, whereas FABPs of other types, namely bovine hFABP and iFABP, virtually show no staining.

Whether other types of fatty acid-binding proteins exist, e.g. renal [11] and skeletal muscle FABP [12], is an unsettled issue at present that has to await sequencing of these proteins. Recently the renal-type FABP, found together with cardiac FABP in rat kidney, was identified as α_{2u}-globulin by Kimura *et al.* [13], whereas gastrotropin appears to be a further type of FABP expressed in mucosal cells of the gastrointestinal tract [2]. In this regard the classification into types seems somewhat questionable as proteins that originally have not been discovered as fatty acid binding proteins, e.g. aP2 and myelin P2 protein, are highly homologous to cFABP and also bind fatty acids.

From terminally differentiated bovine mammary gland a growth regulating factor, termed mammary-derived growth inhibitor (MDGI) was purified by Böhmer *et al.* [14]. Purification is monitored by an *in vitro* proliferation assay using Ehrlich ascites mammary carcinoma cells from the stationary phase of growth *in vivo* and employs $(NH_4)_2SO_4$-precipitation, Sephadex G 50 gelfiltration and DEAE-Sepharose CL 6B anion exchange chromatography. The fraction with inhibitory activity contains a 14.5 kDa polypeptide as shown by SDS-PAGE as well as size exclusion HPLC in the presence of 0.1% SDS [4]. The amino acid sequence of MDGI [4] which is obtained in a N-terminally blocked form, shows virtually no sequence homology to the proliferation inhibitor TGF-beta or any of the structurally known interferons. Instead, a significant homology (95%) is found with the cardiac-type fatty acid-binding protein from bovine heart [15] (Fig. 2) and thus with a family of proteins associated with the differentiated state of cells such as p422, whose cDNA level is increased during 3T3 preadipocyte differentiation [16] and the cellular retinol and retinoic acid-binding proteins. The sequences of bovine cFABP and MDGI differ in only 6 positions, the latter, however, lacks amino acid 132.

In order to obtain MDGI-cDNA for *in situ* hybridisation experiments (see below), a MDGI-specific probe from the cDNA synthesized from RNA of lactating mammary gland was amplified by the polymerase chain reaction (Kurtz *et al.*, in preparation). The amplification with primers complementary to amino acids 16 to 21 and 97 to 101 generated a 258 bp fragment, which then was used to probe the bovine mammary gland cDNA library under conditons of low stringency. The 9 cDNA clones isolated have a length of 680 bp and an identical sequence. The open reading frame codes for most of the amino acids reported for MDGI, but differences are found at 6 positions together with a Met- and an Ala-residue at positions 1 and 133 of the cDNA deduced sequence, respectively. These differences at positions 13, 15, 41, 44, 94 and 128 of the cDNA coincide with those on protein level between MDGI and cFABP, the latter besides having a C-terminal Ala. The differences at position 13 (Asp for Ser), 15 (Lys for Glu), 94 (His for Gln) and 128 (Thr for Val) are designated as less abundant in the reported MDGI-sequence [4]. Thus, the cDNA deduced MDGI-sequence is identical with that reported for the bovine heart cFABP [15] with the exception that 9 terminal nucleotides of the 5′-untranslated region and 4 nucleotides adjacent to the poly-dA tail are deleted in the MDGI clones.

Presence of isoforms

Multiple forms of hepatic-type fatty acid-binding protein are described in the literature [17, 18, 19]. In order to address a certain FABP out of the variety of isoforms and types, the pI of their apoform is used. Particularly in delipidated bovine liver cytosol, two isoforms exist, namely pI 7.0- and pI 6.0-hFABP [17]. Upon lipidation of these apoproteins with fatty acids or their fluorescent derivatives, e.g. 16-(9′-anthroyloxy)palmitic acid (A16 : 0), a shift in isoelectric points is observed. As shown in Fig. 3 the complex of pI 7.0-hFABP with oleic acid (lane 1) focuses at pH 5, whereas the one with A16 : 0 focuses at pH 6 (lane 2). The corresponding complexes of pI 6.0-hFABP both migrate to pH 5 (data not shown). Thus the heterogeneity of hepatic FABPs is a result of different isoforms as well as differences in lipidation. Apart from hFABP, the cytosol of bovine small intestine contains a single form of intestinal-type FABP with an isoelectric point of 5.4 [7]. This latter pI is not affected by ligand binding (Fig. 3, lanes 4, 5).

As mentioned above two isoforms of cardiac-type FABP are present in the cytosol of bovine heart cells [10]. Hitherto no isoforms have been detected in the myocard of other mammals. Cation exchange chromatography of partially purified 15 kDa fractions from bovine and human heart cytosols [8, 10] resolves two peaks of fatty acid-binding activity in the first, but only one single peak in the latter case (Fig. 4). The insert of Fig. 4 shows the isoelectric points of purified proteins as determined by focusing. When charged with radiolabeled fatty acids prior to isoelectric focusing, cardiac FABPs do not change their pI. The comigrat-

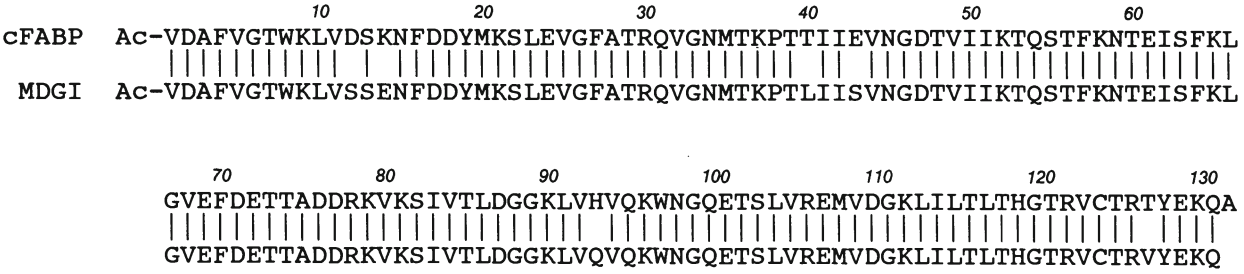

Fig. 2. Sequence relationship between cardiac-type fatty acid-binding protein from bovine heart [15] and bovine mammary-derived growth inhibitor [4].

ing ligands are easily detected by autoradiography indicating their strong affinities to this type of FABP. Recently, Jones *et al.* [20] reported the isolation of two FABP isoforms with pI 4.8 and 4.9, respectively, from rat mammary gland, presumably of the cardiac type. Whether one of these isoforms represents mammary-derived growth inhibitor in this tissue has to be considered. Like for cardiac-type FABP from bovine heart at least two MDGI isoforms differing in their isoelectric points are detected in bovine mammary gland [4]. Whether these forms are coded by different genes is not known.

The results of the sandwich ELISA (Fig. 1C) already indicate that the bovine cFABP isoforms share much more common epitopes than bovine and human cFABP, presumably due to an even higher homology [15, 21, 22]. In fact Unterberg *et al.* [23] recently attributed the molecular origin of bovine cFABP isoforms to a single difference in position 98 of the amino acid sequence, where Asp is found in pI 4.9-cFABP and Asn in pI 5.1-cFABP. A similar difference may at least partly be involved in bovine hFABP diversity (Korf and Spener, unpublished results).

Binding of ligands

In addition of characteristic shifts in isoelectric points upon loading with fatty acids there are yet other peculiarities of the hepatic-type FABPs. While intestinal and cardiac FABPs exhibit a high specificity for fatty acids, hFABP was even discovered as a binding protein for anionic dyes, carcinogens and xenobiotics [6, 18]. Binding of acyl-CoAs, however, seems to be confined to the recently discovered acyl-CoA binding protein [24, 25].

Though the stoichiometry of fatty acid binding to FABPs is still a matter of debate, most investigations,using either the Lipidex- or a liposome-binding assay, found a 2 : 1 ratio for hepatic FABP and a 1 : 1 molar ratio for intestinal and cardiac FABP, respectively. Scatchard analysis allows to separate the isotherms for the binding of the two fatty acids to bovine pI 7.0-hFABP as the affinity of the second is in the order of one magnitude lower than the first [26]. A compilation of binding data is given in Table 1. Furthermore, the circular dichroism induced upon binding of two molecules *trans*-parinaric acid to bovine pI 7.0-hFABP indicates that these fatty acids are located within the same binding site [30]. This concept is supported by a quantitative evaluation of the focusing data presented above. For a complex of pI 7.0-hFABP and radioactively-labeled oleic acid focusing at pH 5, Haunerland *et al.* [17] determined a ligand to protein ratio of 2 : 1. However, from the corresponding complex with 16-(9′-anthroyloxy)-palmitic acid focusing at pH 6 only one mol ligand per mol FABP can be extracted. Obviously the bulky fluorophor of the fatty acid precludes binding of another fatty acid molecule.

Ionic interactions between fatty acid and hFABP can be shown by application of the electrophoretic titration technique [17]. During electrophoresis in a prefocused gel complexes of radioactive fatty acid and pI 7.0-hFABP dissociate below pH 5, most probably due to protonation of the fatty acid. By modification with phenylglyoxal Schulenberg-

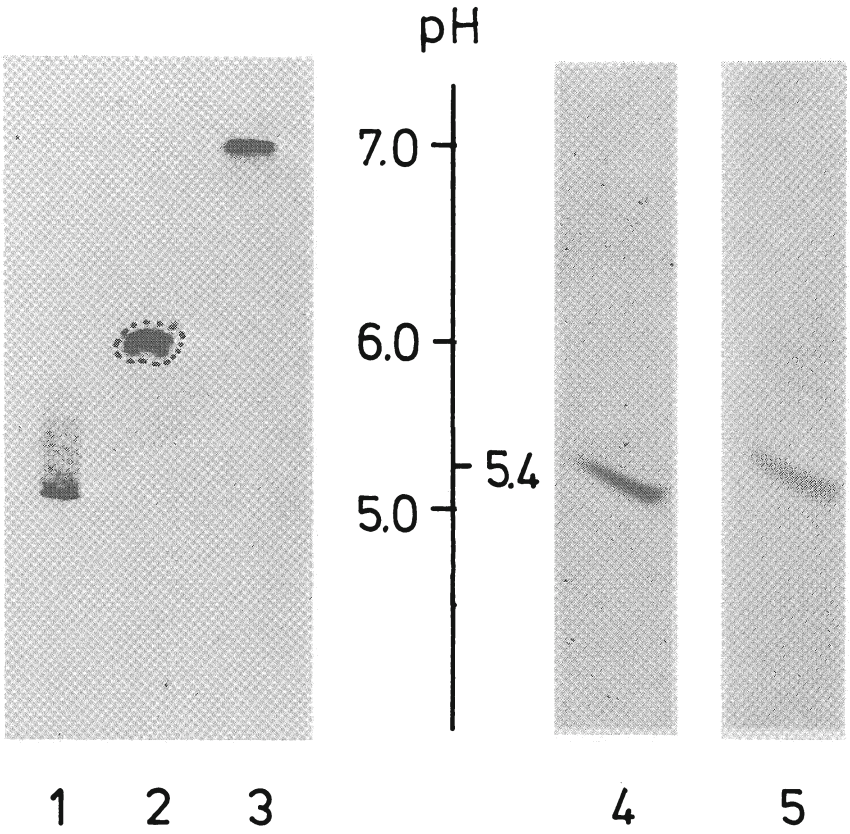

Fig. 3. Isoelectric focusing of fatty acid-binding proteins. 1–3, Bovine pI 7.0-hFABP; 1, loaded with oleic acid; 2, loaded with 16-(9′-anthroyloxy)palmitic acid; 3, delipidated ; 4, 5, bovine pI 5.4-iFABP loaded with [1-^{14}C]oleic acid. 4, Protein stain (Coomassie); 5, autoradiographic track of ligand.

Schell *et al.* [26] then identified arginine as the cationic counterpart of the fatty acyl carboxylate as the binding activity of the modified protein is reduced to 35%. Hence, a similar mechanism may be envisaged for other hepatic- and most probably intestinal-type FABPs, as for the latter X-ray crystallography reveals a close proximity of the fatty acid's carboxylic group to the guanidinium group of Arg^{127} [31].

Fluorescence measurements of complexes of bovine pI 7.0-hFABP and fatty acids, which are substituted with the anthroyloxy group at different positions indicate the contribution of hydrophobic interactions to binding. Blue shifts in emission maxima and increased lifetimes are observed [30], later Storch *et al.* [32] applied this technique with similar results to rat hFABP.

An analysis of putative endogenous MDGI ligands reveals the presence of long chain fatty acids, associated to MDGI prior or during preparation, as well as the capability of MDGI to bind long-chain fatty acids in respective binding assays [33]. The binding constants for the fatty acids were found too low (1 μM) to assume depletion of some essential fatty acids from the medium by MDGI as the reason for growth inhibiton *in vitro*. A synthetic peptide corresponding to the residues 121–131 of the MDGI-sequence exhibits very similar effects as MDGI in a cell proliferation assay (Langen *et al.*, in preparation). This peptide, however, does only bind negligible amounts of fatty acid. Thus, fatty acid binding may not be related to the biological activities described here but may stabilize the MDGI structure and activity because delipidated MDGI undergoes structural changes interpretable as a partial unfolding [34].

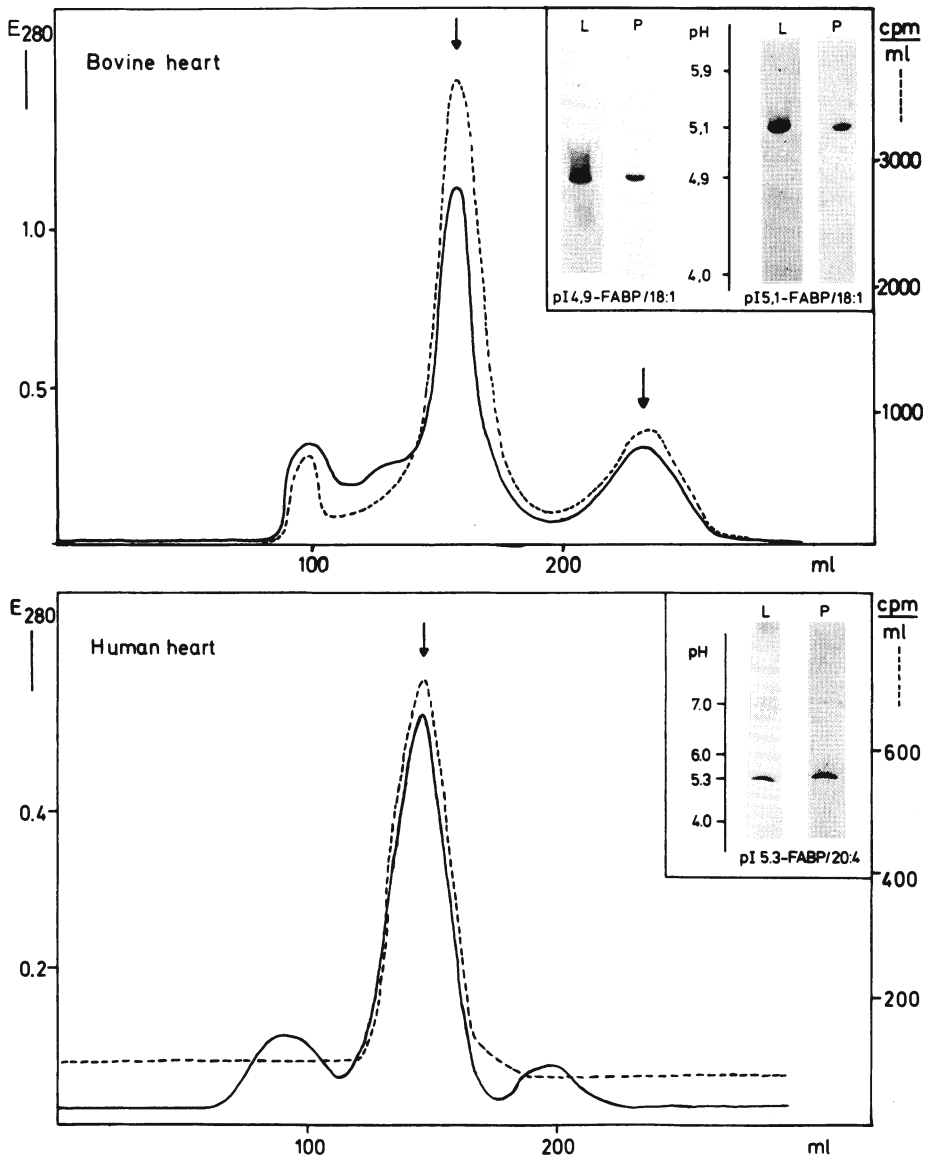

Fig. 4. Cation exchange chromatography of cardiac fatty acid-binding proteins. Partially purified 15 kDa fractions from bovine (upper panel) and human (lower panel) heart cytosols were incubated with [1-^{14}C]oleic acid, applied to a CM-Sephadex C 50 column equilibrated in 20 mM phosphate buffer (pH 6.0) and eluted with the same buffer (12 ml/h). Arrows indicate cFABP-containing peaks. Inserts show isoelectric focusing of these proteins after loading with [1-^{14}C]oleic acid or [1-^{14}C]arachidonic acid. L, Autoradiographic track of ligand; P, protein stain (Coomassie).

Subcellular distribution of fatty acid-binding proteins and mammary-derived growth inhibitor

In order to elucidate the contribution of FABPs to specific metabolic paths of lipids, we and others studied the subcellular distribution of hepatic- and cardiac-type FABP by means of immunomicroscopic [5, 9, 35] and biochemical [5, 9, 36] methods. The elegant protein A-gold method with affinity purified polyclonal antibodies unravels a completely different distribution pattern of FABP-types in liver parenchymal cells and heart myocytes (Fig.

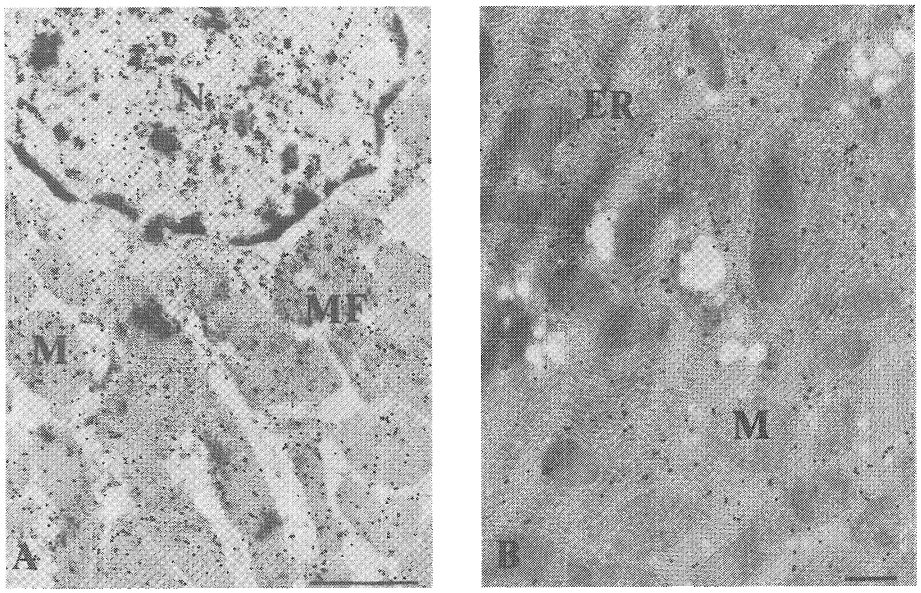

Fig. 5. Immunocytochemical detection of FABP (protein A-gold method) in rat heart and liver cells. N, nucleus; MF, myofibrils; M, mitochondria, ER, endoplasmic reticulum. Panel A, rat heart cell incubated with anti-cFABP-antibodies, × 25500; bar 1 μm. Panel B, rat liver cell incubated with anti-hFABP-antibodies, × 35500; bar 0.5 μm.

5). The electron-micrographs of the liver cell reveal gold-decorated hFABP only in the cytosolic space, preferentially along membranes of endoplasmic reticulum and mitochondria. In contrast to myocytes no staining is observed inside mitochondria and even the Kupffer cells are completely free from label, thus serving as internal controls in those micrographs. Gold-decorated cFABP, however, can be seen within mitochondria and on myofibrils of the heart cell. Moreover, nuclei of both tissues are labeled [5, 9].

Table 1. Binding of oleic acid to fatty acid-binding proteins

Protein	B_{max} [mol/mol]	K_d [μM]	Ref
bovine cFABP[a]	0.93	0.27	
human cFABP	0.45	0.20	[27]
rat cFABP	0.43	0.38	[27]
bovine hFABP	2.1	0.24/2.15[b]	[26]
rat hFABP[c]	1.90	1.77	[28]
rat hFABP	1.2	0.4	[6]
rat hFABP	1.34	1.03	[29]
rat iFABP[c]	0.84	2.87	[28]

[a] pI 4.9-cFABP (Jagschies and Spener, unpublished data).
[b] K_d values for binding of 1st and 2nd ligand molecule to pI 7.0-hFABP from Scatchard plot.
[c] expressed in *E. coli.*

These findings are supported by fractionation of intracellular membranes employing sucrose density gradient centrifugation and subsequent subfractionation of mitochondria after either digitonin treatment or hypotonic swelling. Whole mitochondria and submitochondrial fractions are then analyzed with the aid of a newly developed gelchromatographic immunofluorescence assay, based on the double antibody technique [5]. The membranes are incubated with FABP-specific, affinity purified antibody and, after repeated washings, with an FITC-antibody against the first. Membrane associated fluorescent antibodies are subsequently separated from unbound by gelfiltration on Sephacryl S 1000 (Fig. 6). Control runs without first specific antibody yield a weak fluorescence in the void volume, due to scattering effects of the membranes. In case of intact liver mitochondria an increase in fluorescence is recognized, that is even more pronounced when outer membrane fractions are probed. Liver microsomes react in a similar way in this assay [5]. From our results we conclude that hFABP is associated with these intracellular membranes of liver

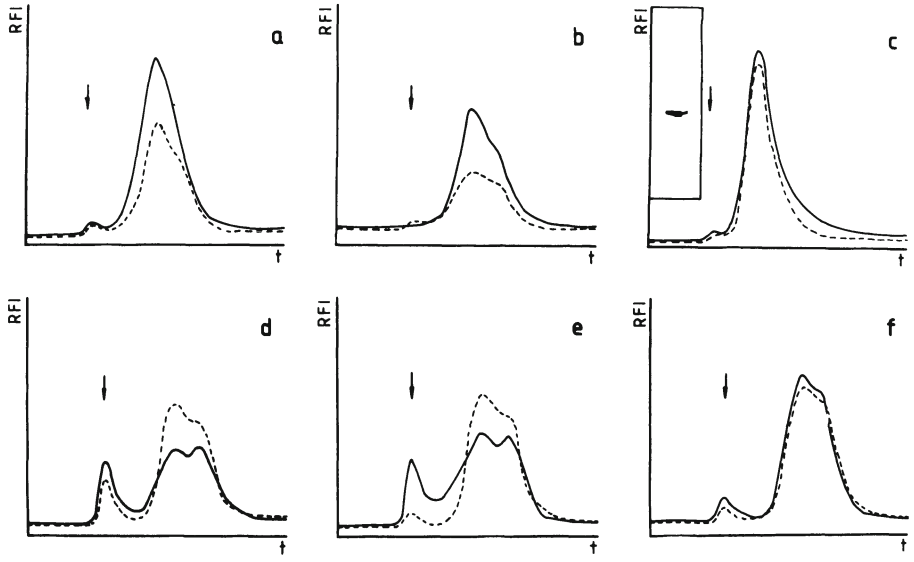

Fig. 6. Gelchromatographic immunofluorescence assay for FABPs in mitochondrial membranes. (——), membranes incubated with FABP specific- and second FITC-labeled antibody; (– – –), control (FABP-specific antibody omitted); t, elution time from Sephacryl S 1000 column (1 × 14 cm); arrow, exclusion volume of the column. a–c, Mitochondria from bovine heart; a, intact mitochondria (1 mg); b, outer membrane (0.5 mg); c, inner membrane (1 mg), insert, western-blot analysis of matrix proteins released after sonication of heart mitoplasts. d–f, Mitochondria from bovine liver; d, intact mitochondria (1.4 mg); e, outer membrane (0.5 mg); f, mitoplasts (0.4 mg).

cells. Heart mitochondrial subfractions, however, exhibit no increase of fluorescence in the void volume, indicating absence of cFABP in these membranes.

Upon sonication of bovine heart mitoplasts, which are essentially free from cytosolic contaminations as ascertained by absence of the cytosolic marker enzyme lactate dehydrogenase [9], a soluble cardiac-type fatty acid-binding protein is released. In the insert of Fig. 6 panel C, the Western blot analysis of matrix proteins clearly demonstrates the presence of this protein. The removal of membrane proteins, which constitute around 90% of mitochondrial proteins is illustrated by the 10-fold increase of cFABP in the mitochondrial matrix (0.18 μg/mg) as compared to whole mitochondria (0.018 μg/mg) [9].

Immunolabeling of mammary epithelial cells with affinity purified antibodies against MDGI is associated with basal invagination, the cytosol and the transcriptionally active euchromatic regions of nuclei, however, at 70 kDa after Western blotting in the latter case [37]. Other cell compartments such as mitochondria and rough endoplasmic reticulum are not labeled.

Biological action of mammary-derived growth inhibitor

Based on findings in Northern blot analysis that transcription of MDGI is related to the proliferative state of mammary gland, cryosections taken from virgin and pregnant cows as well as from different areas of lactating mammary gland are analyzed for MDGI-mRNA using the *in situ* hybridisation technique (Kurtz *et al.*, in preparation). In a developing lobulus MDGI transcription is enhanced in the alveolar cells when compared to ductal epithelial cells (Fig. 7A). Lobuloalveolar structures in glands of a pregnant animal have an increased MDGI-mRNA level in the alveolar epithelial cells bordering on the connective tissue (not shown). In contrast to the pregnant stage both the alveolar and ductal cells of terminally differentiated mammary gland transcribe the MDGI gene

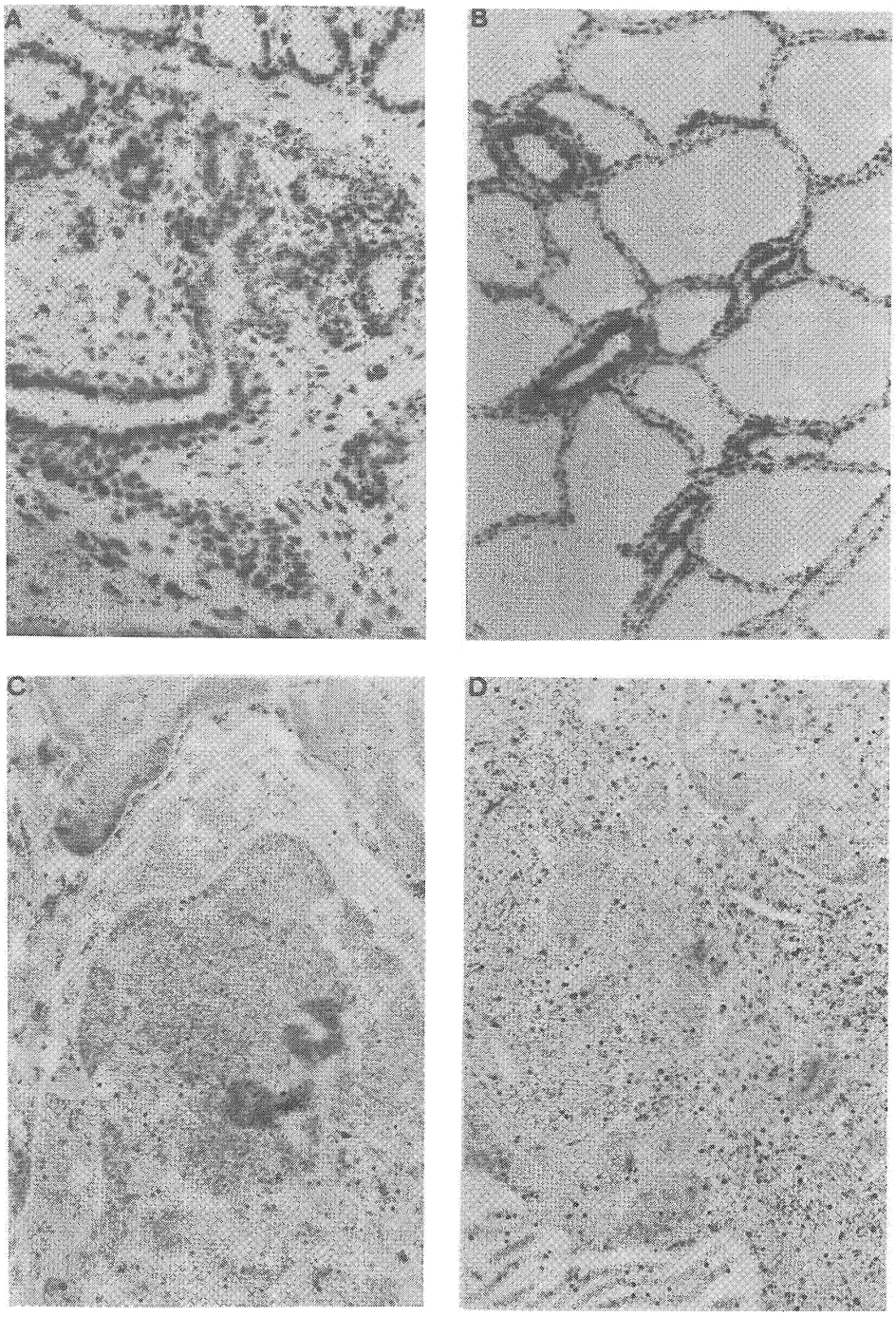

Fig. 7. Distribution of MDGI-mRNA and protein in pregnant and lactating bovine mammary tissue. MDGI-transcripts were detected by *in situ* hybridisation, using cryostat sections hybridized with ^{35}S-labeled antisense RNA probe. A, Longitudinal section of midpregnant mammary gland: B, cross sections of proximal part of lactating mammary tissue; C, Lowicryl K4M embedded sections of pregnant, and D, of lactating mammary gland incubated with anti-MDGI-IgG.

(Fig. 7B). By means of immunohistochemical analysis using affinity purified antibodies MDGI is not espressed in virgin tissue (not shown), whereas a moderate intensity of immunolabeling is found during pregnancy (Fig. 7C). The maximal level is reached during functional differentiation of the mammary gland (Fig. 7D).

Cellular activities are exerted on Ehrlich ascites carcinoma cells as well as on mammary epithelial cell lines. Dose-response curves for inhibition of proliferation of a hyperdiploid line of 'stationary Ehrlich cells' by purified MDGI show a half maximal inhibition at 1 ng/ml. Furthermore, cells become insensitive to inhibition by preincubation for 4 h with serum. Likewise, inhibition is abolished by simultaneous addition of MDGI with EGF or insulin. The inhibitory activity of MDGI is also antagonized by 10 μM 2'-deoxycytidine. Ehrlich cells are shown to possess PDGF receptors (Böhmer, unpublished results). An antagonistic PDGF effect is only observed if the cells are pretreated with 3–10 ng/ml PDGF 4 h before addition of MDGI. The PDGF effect proceeds even if PDGF is washed out before addition of MDGI. Insulin or EGF do not prevent growth inhibition if added to Ehrlich cells during the preincubation period. In summary, the data indicate that MDGI is acting by reducing the rate the cells are passing through some restriction point in G1/S.

The response of permanent mammary carcinoma cell lines (MATU, MCF-7) and normal human mammary epithelial cells to MDGI is based on a serum starvation to trigger cells into quiescence [38] followed by a restimulation with fresh medium. MDGI is present during the restimulation period for 16–20 h. Flow cytophotometric measurements with MATU, MCF-7 and normal human mammary epithelial cells proved them to be arrested in G1/G0. Obviously, synchronization of the cells in G1/G0 is a prerequesite to measure growth inhibition in presence of MDGI as cells not arrested by serum starvation are not responsive. DNA-synthesis in all but MCF-7 cells can be partially blocked by MDGI. For MATU cells the antgonistic effect of insulin can be confirmed (see above). Antisera raised against MDGI have a neutralizing effect on growth inhibition by MDGI in both, the Ehrlich ascites carcinoma cells [14] and in the mammary carcinoma cell line MATU [4]. In summary, MDGI is a growth regulating peptide with different functions including growth arrest during normal development of tissues and organs. The presented data may suggest that MDGI transcription is related to some functional role of MDGI for the onset of early differentiation which is coupled to inhibition of cell proliferation. At present work is in progress to further disclose the functional relationship between cardiac-type fatty acid-binding protein and mammary-derived growth inhibitor.

Acknowledgement

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Expression of a functionally active cardiac fatty acid-binding protein in the yeast, *Saccharomyces cerevisiae*

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Key words: bovine heart fatty acid-binding protein, H-FABP$_c$, heterologous gene expression, *Saccharomyces cerevisiae, GAL10* promoter

Summary

The unicellular eukaryotic microorganism, *Saccharomyces cerevisiae,* transformed with a plasmid containing a cDNA fragment encoding bovine heart fatty acid-binding protein (H-FABP) under the control of the inducible yeast *GAL10* promoter, expressed FABP during growth on galactose. The maximum level of immunoreactive FABP, identical in size to native protein as judged from SDS-polyacrylamide gel electrophoresis, was reached after approximately 16 hours of induction. Analysis of particulate and soluble subcellular fractions showed that FABP was exclusively associated with the cytosol. FABP expressed in yeast cells was functional as was demonstrated by its capacity to bind ^{14}C-oleic acid in an *in vitro* assay. Growth of the transformants on galactose as the carbon source was significantly retarded at 37° C. Whereas the fatty acid pattern of total lipids was not altered in transformed cells, desaturation of exogenously added ^{14}C-palmitic acid was significantly reduced both at 30 and 37° C. The lowest percentage of radioactively labeled unsaturated fatty acids was found in the phospholipid fraction.

Introduction

Fatty acid-binding proteins (FABPs) represent a highly abundant species of low molecular weight proteins present in the cytosol of many mammalian and plant tissues [1]. Although many effects of these proteins have been detected *in vitro,* their physiological significance remains unclear. The availability of cDNA clones encoding cardiac, hepatic, or intestinal FABP from different species now allows the function of these proteins to be analyzed on a molecular basis.

The unicellular eukaryote *Saccharomyces cerevisiae* (baker's yeast) shares many of the basic routes of fatty acid biosynthesis and lipid metabolism with multicellular organisms. However, yeast does not contain proteins which are functionally or structurally homologous to the fatty acid-binding proteins of higher eukaryotes. Thus, expression of a functional, heterologous FABP in *S. cerevisiae* should provide some information about the physiological role of these proteins with respect to the intracellular fatty acid transport and metabolism.

In this report we describe the cloning of a H-FABP-encoding cDNA in *Saccharomyces cerevisiae* and the effect of functional FABP on cellular growth, fatty acid composition, and intracellular utilization of exogenously added fatty acids.

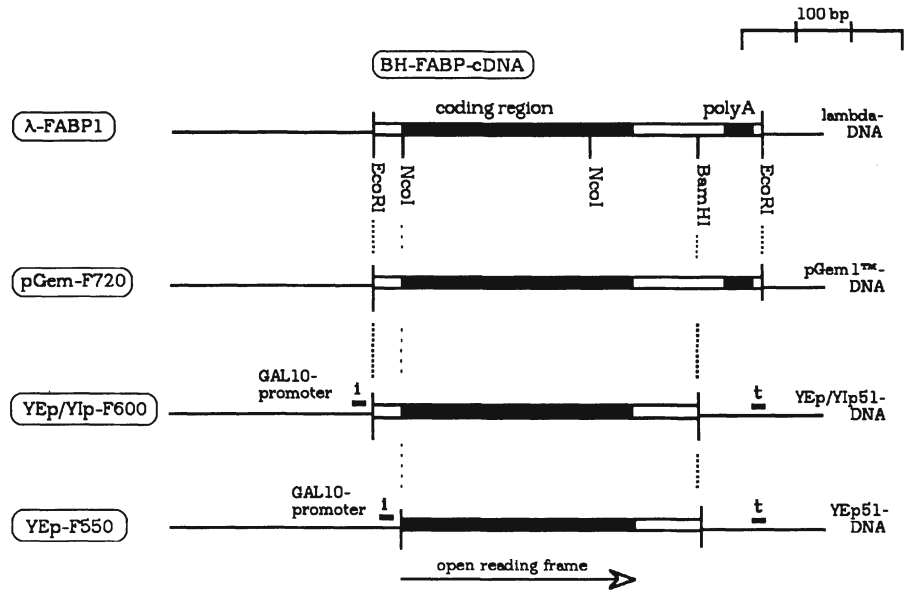

Fig. 1. Construction of yeast FABP-expression plasmids. An EcoRI fragment containing a full-length cDNA encoding bovine heart FABP (BH-FABP$_c$) was cloned from λgt11 (λ-FABP1) into pGEM®-1 (pGEM-F720). Smaller fragments were subcloned from pGEM-F720 into YEp51 for expression of the FABP gene under the control of the yeast *GAL10* promoter (YEp-F600 and YEp-F550). Integrating plasmid YIp-F600 was created by deleting the 2 μ origin of replication from YEp-F600. For details, see Experimental Procedures. i: initiation of transcription; t: termination of transcription.

Experimental procedures

Strains and culture conditions. *E. coli* strain MC 1066 (*F⁻ Δ(lac)X74 hsr⁻ hsm⁻ rpsL galU galK trpC9830 leuB600 pyrF::Tn5*) was used for cloning, maintenance and propagation of plasmids. Ampicillin was added to the media at a concentration of 100 μg/mL to maintain selective pressure. *Saccharomyces cerevisiae* strain W303-1A (*MATa leu2-3,112 his3-11,15 ade2-1 ura3-1 trp1-1 can1-100,* provided by R. Rothstein) was used for transformation and FABP-gene expression. Cells were cultivated in liquid media containing all nutrients except leucine to prevent loss of the episomal or integrated plasmids. Glucose or galactose were added as the carbon source at a concentration of 3%, as indicated.

Yeast and bacterial transformations, DNA manipulations and cloning procedures were done following standard procedures [2]. Enzymes for DNA manipulations were purchased from Boehringer, Mannheim.

Construction of yeast expression plasmids containing H-FABP-cDNA inserts (Fig. 1). The EcoRI fragment of λgt11 vector λ-FABP1 containing a 720 bp H-FABP-cDNA insert [3] was cloned into the respective site of plasmid pGEM®-1 (Promega Biotec) to yield plasmid pGEM-F720. A 600 bp EcoRI (blunt ended with Klenow polymerase)/BamHI fragment lacking the 3'polydA-tail of the cDNA was isolated and inserted into the SalI (blunt ended with Klenow polymerase)/BclI sites of yeast expression vector YEp51. YEp51 contains the *GAL1/GAL10* promoter, a transcription termination site, the 2 μ origin of replication and a yeast *LEU2* selectable marker (provided by J. Broach). In this construction, YEp-F600, the *GAL10* promoter lies 46 bp upstream of the translation initiation site. In YEp-F550, 45 bp between yeast upstream sequences and the translation initiation site were deleted (Fig. 1). By deleting the XbaI/HpaI fragment containing the origin of replication, an integrating version of vector YEp-F600 was constructed (YIp-F600). In all expression studies,

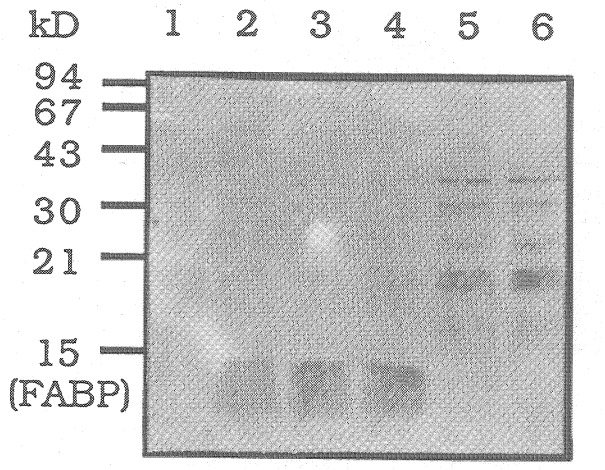

Fig. 2. Cell fractionation and immunological analysis of FABP. Proteins were isolated from transformants harboring plasmid YEp51 without an insert (lane 1 and 5) or FABP-cDNA-containing plasmids YEp-F600 (lane 2 and 6), YIp-F600 (lane 3) or YEp-F550 (lane 4). The proteins were separated into soluble (lane 1–4) and particulate (lane 5–6) fractions before analysis by PAGE and immunoblotting with anti FABP antibodies.

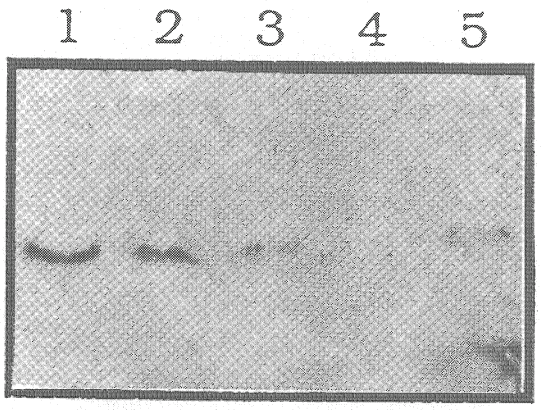

Fig. 3. Binding of fatty acids *in vitro*. Proteins were incubated with [1-^{14}C] oleic acid and separated by isoelectric focussing. Autoradiography of the dried gel was used to detect those proteins that had bound the labeled fatty acid. Lanes 1 and 2: FABP isolated from beef heart in phosphate buffer and the buffer used for yeast protein preparation, respectively. Lanes 3 and 4: cytosol from cells transformed with control plasmid YEp51, either with [3] or without [4] the addition of FABP during the incubation. Lane 5: cytosol from cells transformed with plasmid YEp-F600.

wild-type cells (W303-1A) transformed with vector YEp51 without insert were used as a control.

Cell fractionation and immunological analysis of FABP. Yeast transformants, grown on media containing 3% galactose to an OD_{548nm} of 4.5–5, were harvested by centrifugation and broken with glass beads in a Braun-Melsungen homogenizer under CO_2-cooling. Cell debris was removed by centrifugation, and soluble and membrane fractions were separated by centrifugation at 100,000 × g for one hour. SDS-polyacrylamide gel electrophoresis of soluble and membrane proteins (30 μg protein per lane) was performed as described by Laemmli [4]. After separation of proteins and transfer to nitrocellulose filters, FABP was detected by immunodecoration with rabbit-anti bovine heart-FABP-antibody and peroxidase-conjugated goat-anti rabbit-antibody.

Binding of fatty acids *in vitro*. Cytosolic fractions from transformed strains (30 μg) and preparations containing authentic FABP isolated from bovine heart (200 ng) were incubated with [1-^{14}C] oleic acid for 30 min at 30° C. Samples were subjected to isoelectric focusing as described [5], and the dried gels were exposed to Kodak X-Omat X-ray film for autoradiography.

Labeling of cells with ^{14}C-palmitic acid, lipid extractions and analyses. Transformants were grown over night at 30 or 37° C on media containing the respective carbon source to an OD_{548nm} of 4.5–5. Cells were harvested by centrifugation and resuspended at a concentration of 1 mg/mL in fresh media. After preincubation at the respective temperature (one hour), [1-^{14}C] palmitic acid (59 mCi/mmol) was added to a final concentration of 5 μM, and the cells were incubated for one hour. Non-internalized fatty acid was removed by washing cells in the presence of 0.1% fatty acid-free bovine serum albumin. Lipids were extracted following the protocol of Folch [6] and separated by one-dimensional thin-layer chromatography on silica-gel H60 plates, using petroleum ether/diethylether/glacial acetic acid 80 : 20 : 2 [per vol.] as the developing solvent. Fatty acid methyl esters were prepared by transesterification with boron trifluoride/methanol from total lipids or from phospholipids isolated by preparative thin-layer chromatography. Saturated and unsaturated fatty acid methyl esters were separated by argentation TLC using the

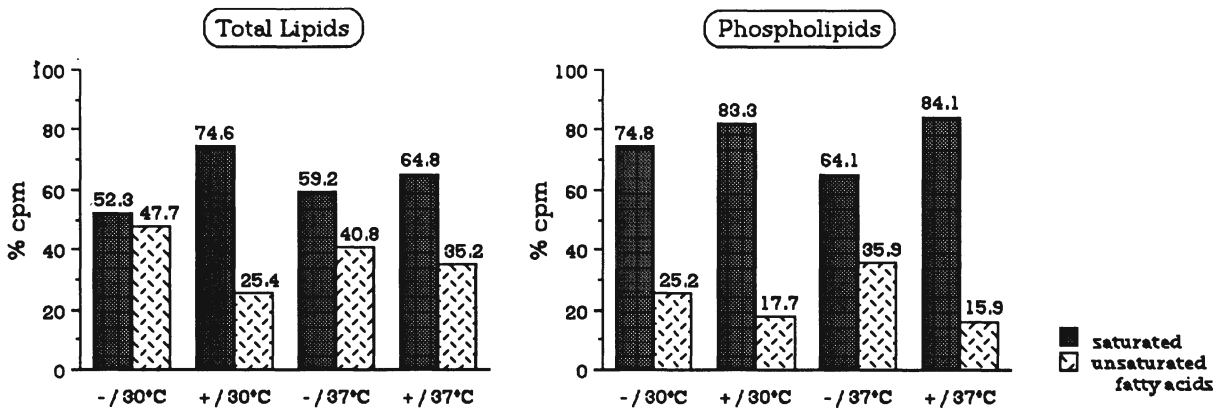

Fig. 4. Labeling of cells *in vivo* with ^{14}C-palmitic acid. Cells at mid-log phase were incubated with [1-^{14}C] palmitic acid, and their lipids were extracted. Fatty acid methyl esters were prepared from total lipids and from phospholipids. Saturated and unsaturated methyl esters were separated by argentation TLC. Radioactivity was measured by liquid scintillation counting. The results are the mean of two independent experiments with a deviation of less than 10%.
− : transformants not expressing FABP (YEp51).
+ : transformants expressing FABP (YEp-F600).

developing solvent petroleum ether/diethylether 90 : 10 [per vol.] [7].

Radioactivity in whole cells and individual lipid fractions was determined by liquid scintillation counting in a Tri-Carb 1500 LSC from Canberra Packard.

Results

Yeast cells transformed with plasmid YEp-F600 containing bovine heart FABP-cDNA under control of the yeast *GAL10* promoter (Fig. 1) expressed fatty acid-binding protein during growth on galactose as the sole carbon source. The maximum level of FABP was reached after approximately 16 hours of induction. Cell fractionation by differential centrifugation and analysis of fractions by SDS-polyacrylamide gel electrophoresis and immunoblotting showed that the protein was present only in the cytosolic fraction (Fig. 2), where it accounted for approximately 0.5–1% of total protein. The quantities of cross-reactive proteins in the membrane fraction were the same in control and FABP-expressing cells.

Transfer of cells into glucose medium resulted in a rapid disappearance of FABP with a half life of less than one hour. The maximum level of protein was approximately the same in cells harboring the episomal multicopy plasmid YEp-F600 or the integrated vector YIp-F600, indicating that copy number of the FABP gene did not affect the expression rates. Decreasing the distance between the translation initiation site and the *GAL10* promotor by 45 bp in YEp-F550 also had no effect on the expression of the FABP gene (Fig. 2).

According to SDS-polyacrylamide gel electrophoresis, the FABP expressed in yeast has the same size as the protein isolated from beef heart. Its functionality was demonstrated by its capacity to bind [1-^{14}C] oleic acid (Fig. 3). In a first attempt to assess possible effects of FABP on cellular fatty acid metabolism, the fatty acid compositions of total lipids and individual glycerolipid classes isolated from cells transformed with the control plasmid YEp51 and transformants expressing FABP were compared. The FABP did not affect either the phospholipid composition or the fatty acid pattern of neutral lipids or of total phospholipids. However, a marked effect of FABP on the utilization of exogenous [1-^{14}C] palmitic acid was apparent. After incubating cells with trace amounts of [1-^{14}C] palmitic acid for one hour, the total amount of radioactively labeled fatty acid incorpo-

rated into glycerolipids was essentially the same in control cells and cells expressing FABP. However, the degree of unsaturation of the exogenously added fatty acid was significantly different. Data in Fig. 4 show a markedly reduced percentage of radioactivity associated with unsaturated fatty acids in FABP expressing cells. The difference in the phospholipid fraction was more pronounced when cells were grown at 37° C. At this temperature the growth rate of transformed cells was also significantly retarded.

Discussion

The rationale for expressing FABP in yeast cells, which normally do not contain these or analogous proteins, is to test whether one of the effects of FABP observed previously in cell-free systems can be confirmed under more physiological conditions *in vivo*.

Yeast cells transformed with the episomal plasmid YEp-F600 or the integrating vector YIp-F600, both containing cDNA encoding FABP from beef heart, expressed the protein at a level of about 0.5–1% of cytosolic protein, which is approximately the same as the concentration in beef heart cytosol [8]. The FABP level in yeast cells is most likely limited by rapid proteolytic degradation. Yeast cells expressing FABP show no phenotype when grown at 30° C, but their growth rate was significantly reduced at 37° C. The reason for retarded growth at elevated temperatures is not clear. It can be excluded that growth retardation is a result of the presence of the yeast *GAL10* promoter in high copy number because wild-type cells transformed with the plasmid containing *GAL10* promoter and no insert grew normally in the presence of 3% galactose at this temperature. Furthermore, the same reduced growth was observed if the FABP gene was present in single copy (YIp-F600).

Analyses of the glycerolipid composition and fatty acid pattern of lipids extracted from transformed and control cells gave identical results. Thus it appears that FABP does not influence the biosynthesis of fatty acids and the incorporation of endogenous fatty acids into complex lipids. Further experiments will be necessary to elucidate the relationship between retarded growth and FABP expression.

A specific role attributed to FABPs is that of a carrier participating in the uptake and intracellular transport of exogenous fatty acids. Thus FABPs may not only facilitate entry of fatty acids into cells, but also direct them to or keep them away from distinct intracellular destinations, such as specific subcellular compartments or enzyme systems [9]. In line with such considerations is the observation that exogenously added palmitic acid is less effectively converted to unsaturated fatty acids (palmitoleic acid and, after elongation, oleic acid) in FABP-expressing cells. The distribution of total radioactivity among glycerolipid classes is the same in control and FABP-expressing cells. Further experiments will concentrate on the effect of FABP on the kinetics of fatty acid transport into cells and on the utilization of exogenous fatty acids under different physiological conditions, such as anaerobiosis or when fatty acids are provided as the major source of lipid acyl chains.

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Expression of fatty acid-binding protein from bovine heart in *Escherichia coli*

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Key words: bovine cardiac fatty acid-binding protein, cDNA, expression vector

Summary

The coding part of the cDNA of cardiac fatty acid-binding protein (cFABP) from bovine heart was cloned into the vector pKK233-2. After induction with isopropyl-β-D-thiogalactopyranoside cFABP was found in a soluble form in the cytosol of plasmid transformed *E. coli* amounting up to 5.7% of the soluble protein. cFABP was detected after SDS-polyacrylamide gelelectrophoresis and/or isoelectric focusing and Western blot by immuno-staining and was determined quantitatively by a solid phase enzyme-linked immuno sorbent assay. The cFABP produced by bacteria binds oleic acid with high affinity as shown by comigration of protein and ligand in both gelfiltration and isoelectric focusing. cFABP was purified from bacterial lysates to near homogeneity and resolved into four isoproteins.

Introduction

The cardiac-type fatty acid-binding proteins (cFABPs) belong to a group of well characterized 15 kDa proteins. They bind fatty acids with high affinity [1] and appear to be implicated in intracellular transport and metabolism of long-chain fatty acids [2]. Two isoforms of cFABP differing in their isoelectric points, pI 4.9 and pI 5.1, respectively, exist in bovine cardiac tissue [3]. By cloning and sequencing of a full length cDNA the primary structure of bovine cFABP was elucidated [4]. There remained, however, the question of whether cFABP can be expressed in *E. coli* after cloning the cDNA in an expression vector. Here we report on the construction of an expression vector for bovine cFABP and the purification of the protein from bacterial lysates. We present evidence that the microbially produced cFABP is active, i.e. it binds oleic acid with high affinity.

Experimental procedures

Bacterial strains and vectors. Except for the cFABP cDNA cloned in λgt11 (λ-FABP) by Billich *et al.* [4] commercially available bacterial strains (JM 105) and vectors, pKK233-2 from Pharmacia and pGEM3 from Promega Biotec were used throughout this work. Details of the procedure of recloning the coding sequence into the expression vector are given by Wissel [5].

Growth of cFABP-producing E. coli. Bacteria were cultured under shaking at 37° C in TB Medium (1.2% w/v Bacto Tryptone; 2.4% w/v yeast extract; 0.4% v/v glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) enriched with 50 μg/ml ampicillin, starting from a 5% inoculum. At optical density OD_{550} = 0.2 isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 10 mM and growth was continued up to the stationary phase. The bacteria were harvested by centrifu-

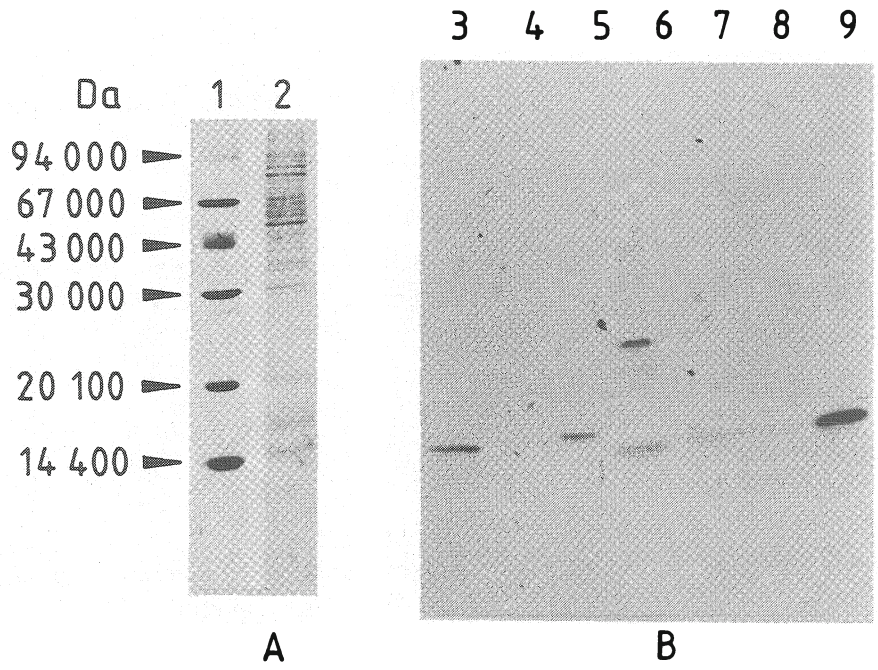

Fig. 1. Detection of cFABP in cell lysates by Western blotting. A, Protein staining; B, immunological detection. 1, Marker proteins; 2, 9, clone pBH-FABP; 3, clone pFABP-2; 4, clone pKK233-2 (negative control); 5, clone pFABP-N; 6, clone pFABP-H; 7, 8, cFABP from bovine heart.

gation at 5 000 × g. The pellet could be stored frozen over several weeks.

Extraction of cFABP from bacteria. A dense suspension of bacteria in 1–2 ml of 20 mM potassium phosphate buffer, pH 6.2, enriched with 1 mM 2-mercaptoethanol and 1 mM phenyl methyl sulfonyl fluoride was sonicated 3 times for 15 s with the Branson sonifier at position 3 using a microtip. Care was taken to keep the temperature below 10° C. The lysate was cleared by centrifugation for 5 min at 14000 × g. The supernatant was used immediately or stored frozen for several days.

Detection and determination of cFABP. Immunostaining of cFABP fixed on nitrocellulose membranes and quantitation of cFABP by an enzyme-linked immuno sorbent assay (ELISA) was carried out as described by Börchers *et al.* [6].

Results

Construction of an expression vector for bovine cFABP

Since the translation start in the cDNA for bovine cFABP lies within a restriction site for endonuclease NcoI the vector pkk233-2 (Pharmacia) seemed most suitable for the construction of an expression vector for cFABP. In this vector transcription is controlled by the strong trc promotor and the start codon is positioned in optimal distance from a ribosome binding site. Unfortunately, the cDNA to be transformed has a second NcoI site 42 nucleotides upstream from the translation stop. Therefore, the construction had to include more than one step.

First a 594 bp fragment of the original cDNA from λ-FABP [4] was obtained by an EcoRI/Bam HI cut and recloned into the vector pGEM3. The resulting vector pGEM594 contained the complete coding sequence for cFABP between the 5′-non-coding and the 149 nucleotides containing 3′-non-

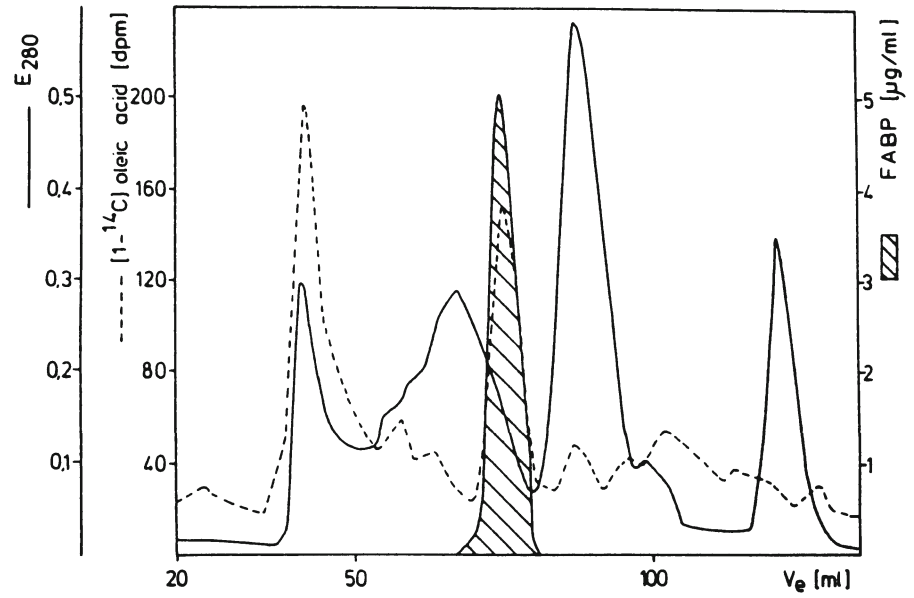

Fig. 2. Coelution of the complex of recombinant cFABP with [1-^{14}C]oleic acid in gelfiltration (Sephacryl S 200). A lysate of pBH-FABP-transformed and IPTG-induced bacteria was incubated with [1-^{14}C]oleic acid and applied onto the column. Radioactivity bound to cFABP (---) coincides with immunoreactive material (hatched area, quantitative determination by cFABP-specific ELISA.

coding sequence. Second, the largest of three NcoI fragments of the cDNA that codes for cFABP starting with methionine and ending 14 amino acids before the translation stop was inserted into PKK 233-2. The resulting vector was called pFABP-N. Two other vectors were obtained by cloning the NcoI insert into the NcoI/HindIII digested PKK233-2. The doubly digested vector was ligated with one or two NcoI fragments. After filling the 5'-ends with DNA polymerase I the vector was closed. The resulting vectors pFABP-H and pFABP-2 differ by harboring one and two NcoI fragments, respectively. The vectors pFABP-N, pFABP-H and pFABP-2 code for carboxy-terminally modified cFABP that were expressed after induction in transformed *E. coli* and were visualised by ELISA after SDS-polyacrylamid gelelectrophoresis (SDS-PAGE) of a lysate and Western blotting (Fig. 1). Third, a KpnI/HindIII fragment which spaces from nucleotide 65 within the coding region to the cloning site of plasmid was replaced from pFABP-N by the corresponding fragment from pGEM594 thus completing the coding sequence. The resulting vector pBH-FABP contains the coding sequence for cFABP starting with a methionine in an optimal position to a ribosome binding site and 149 nucleotides of the 3'-noncoding region of the cDNA. The last step of the cloning was controlled by Sanger sequencing (data not shown).

Synthesis of cFABP in transformed E. coli

pBH-FABP transformed *E. coli,* strain JM 105, were induced with IPTG at the end of logarithmic growth and harvested after reaching the stationary phase. In lysates of transformed and induced bacteria an additional protein at 15.2 kDa can be detected after SDS-PAGE and staining with Coomassie blue. After electroblotting to nitrocellulose this protein reacts with rabbit anti-cFABP-antibody and can be visualized by ELISA with peroxidase antirabbit-antibody-conjugate (Fig. 1). Eventually, a second band is detected corresponding to 30 kDa that is most probably a dimer of cFABP.

In order to assert its identity with cFABP a cleared lysate was incubated with [1-^{14}C]oleic acid and chromatographed on a column of Sephacryl S 200. This procedure separates protein-bound from un-

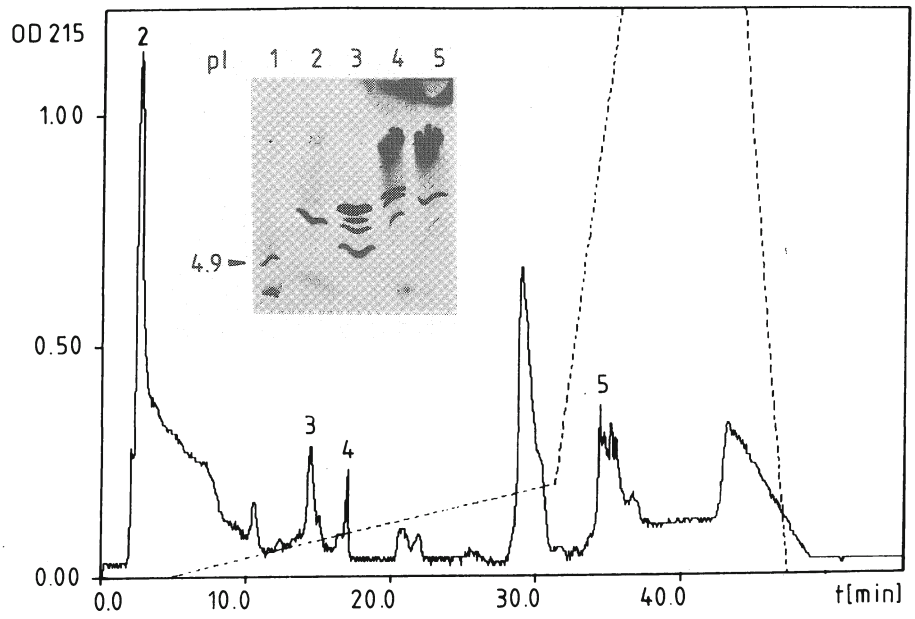

Fig. 3. Anion-exchange FPLC of the S 200-fraction (Fig. 2) on Mono Q HR 5/5. Starting buffer 20 mM Tris/HCl, pH 8.2; concentrated S200-fraction in 500 µl starting buffer was bound to the column and eluted with 0.75 ml/h by KCl-gradient (---). Fractions 2 to 5, containing recombinant cFABP, were collected separately, charged with [1-^{14}C]oleic acid and subjected to IEF. Insert: Autoradiographic tracks of the radioactive ligand after IEF. 1, pI 4.9-cFABP from bovine heart; 2, 3, 4, 5, recombinant cFABP-fractions.

bound fatty acid according to Jagschies *et al.* [3]. In each fraction cFABP was determined by the quantitative ELISA designed for cFABP by Börchers *et al.* [6] as well as oleic acid by scintillation counting. As seen in Fig. 2 oleic acid exactly comigrates with a protein responsive to anti-cFABP-antibody.

Rate of expression and localization in bacteria

The amount of cFABP expressed in *E. coli* JM 105 after induction with IPTG made up to 5.7 ± 0.4% of total soluble protein as determined by the quantitative ELISA. Less than 5% of total cFABP was found in the debris fraction after lysis. It is concluded that the cFABP produced in *E. coli* is present in a soluble form and not as insoluble inclusion bodies. In contrast, carboxy-terminally modified cFABP, e.g. that is expressed by clone pFABP-N, is produced at rates two orders of magnitude less and most of the anti-cFABP-antibody responsive protein is found in the insoluble debris fraction.

Purification of cFABP from bacterial lysates

The purification of cFABP from bacteria follows the procedure elaborated by Unterberg *et al.* [7] for the isolation of cFABP from cardiac muscle.

Chromatography on CM-Sephadex. This was found to be the key step where the bulk of bacterial proteins was removed. The cleared bacterial lysate was applied to a column of CM-Sephadex previously adjusted to 20 mM potassium phosphate buffer, pH 6.2. The column was washed with the same buffer and cFABP was eluted immediately behind the wash-through fraction. The cFABP containing fractions, as judged by simple dot blot ELISA, were pooled and concentrated by ultrafiltration. At this stage the purity of the cFABP was about 40% as estimated from SDS-PAGE.

Gelfiltration on Sephacryl S 200. This step removed most of the remaining contaminants. However, heavy losses were observed sometimes due to unspecific adsorption to the gel. cFABP eluted in the

non-excluded fraction in a simple peak. The cFABP containing fractions were pooled and concentrated by ultrafiltration. At this stage the cFABP was at least 80% pure.

Resolution by FPLC. A final degree of purification was achieved by FPLC on a Mono Q HR 5/5 column (Pharmacia). A 100 µl aliquot equilibrated with 20 mM Tris-HCl buffer, pH 8.6, was applied to the column previously washed with the same buffer. Protein was eluted by a linear gradient from 0–100 mM KCl and the absorbance at 215 nm of the eluate was monitored. As shown in Fig. 3 several peaks containing cFABP were collected and checked for [1-^{14}C]oleic acid binding by isoelectric focusing (IEF) (insert in Fig. 3.). The retention times of peak 3 and 4 correspond closely to those of pI 4.9- and pI 5.1-cFABP.

Discussion

One objective of this study was to build up a biological system that can produce cFABP in larger amounts for structural and physico-chemical studies and avoids the laborious procedure of isolation from mammalian tissue. Vectors for the expression of rat liver FABP and rat intestinal FABP in *E. coli* were described by Lowe *et al.* [8, 9]. The rates of expression of the FABPs by these vectors are comparable to that of the system reported here. It might be possible to improve the yields by manipulation of promotor, ribosome binding site or by optimizing the codon usage. The presence of FABP isoforms in the bacterial lysates was a surprise and poses some problems in the preparation of homogeneous cFABP. Work is in progress to elucidate the posttranslational processes leading to four isoforms.

The expression of cFABP in *E. coli* offers the opportunity to design modified forms of the protein by standard methods of *in vitro* mutagenesis and to study the effect of amino acid exchange on structure and on ligand affinity.

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Expression of rat intestinal fatty acid binding protein in *E. coli* and its subsequent structural analysis: a model system for studying the molecular details of fatty acid-protein interaction

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Key words: fatty acid binding protein, prokaryotic expression vectors, x-ray crystallography

Abstract

A prokaryotic expression vector containing the *rec A* promoter and a translational enhancer element from the gene 10 leader of bacteriophage T7 was used to direct efficient synthesis of rat intestinal fatty acid binding protein (I-FABP) in *E. coli.* Expression of I-FABP in *E. coli* has no apparent, deleterious effects on the organism. High levels of expression of I-FABP mRNA in $supE^+$ strains of *E. coli,* such as JM101, is associated with suppression of termination at its UGA stop codon. This can be eliminated by using a $supE^-$ strain as MG1655 and by site-directed mutagenesis of the cDNA to create an in frame UAA stop codon. *E. coli*-derived rat I-FABP lacks its initiator Met residues. It has been crystallized with and without bound palmitate. High resolution x-ray crystallographic studies of the 131 residue apo- and holo-proteins have revealed the following. I-FABP contains 10 anti-parallel β-strands organized into two orthogonally situated β-sheets. The overall conformation of the protein resembles that of a clam – hence the term β-clam. The bound ligand is located in the interior of the protein. Its carboxylate group forms part of a unique five member hydrogen bonding network consisting of two ordered solvent molecules as well as the side chains of Arg^{106} and Gln^{115}. The hydrocarbon chain of the bound C16:0 fatty acid has a distinctive bent conformation with a slight left-handed helical twist. This conformation is maintained by interactions with the side chains of a number of hydrophobic and aromatic amino acids. *Apo*-I-FABP has a similar overall conformation to *holo*-I-FABP indicating that the β-clam structure is stable even without bound ligand. The space occupied by bound ligand in the core of the holo-protein is occupied by additional ordered solvent molecules in the apo-protein. Differences in the side chain orientations of several residues located over a potential opening to the cores of the apo- and holo-proteins suggest that solvent may play an important role in the binding mechanism. Comparison of the Cα coordinates of apo- and holo-I-FABP with those of other proteins indicates it is a member of a superfamily that currently includes (i) 10 mammalian intracellular lipid binding proteins, (ii) the photoactive yellow protein from the purple photoautotrophic bacterium *Ectothiorhodospira halophila* and (iii) a group of extracellular lipid binding proteins from a diverse number of phyla that have a common β 'barrel' consisting of 8 anti-parallel β-strands stacked in two nearly orthogonal sheets. In summary, *E. coli*-derived I-FABP not only represents a useful model for assessing the atomic details of fatty acid-protein interactions and the mechanisms which regulate acquisition and release of this type of ligand, but also structure/function relationships in other superfamily members.

Abbreviations: I-FABP – Intestinal Fatty Acid Binding Protein, r.m.s. – root mean square

Introduction

Rat intestinal fatty acid binding protein (I-FABP) is a 131 amino acid polypeptide[1] synthesized in at least two of the terminally differentiated cells which populate the small intestinal epithelium: (i) the polarized enterocyte which is the principal absorptive cell of the gut and (ii) the goblet cell which produces mucin [1]. Immunocytochemical studies have shown that I-FABP is located in the cytoplasm of these cells [1, 2]. This intracellular location is also consistent with the observation that the primary translation product of rat I-FABP mRNA contains no cleavable signal peptide or signal peptide equivalent that would direct it to the secretory apparatus [3]. I-FABP gene expression appears confined to the small intestine of rodents (mice and rats) and nonhuman primates [4]. Distinct regional differences in I-FABP mRNA and protein accumulation have been documented within the small intestinal epithelium of fetal, neonatal and adult rats and mice [1, 5]. The protein is not detectable until differentiating epithelial cells exit the crypts of Lieberkühn and begin their translocation up the villus [1]. Moreover, along the proximal to distal axis of gut (i.e. from duodenum to ileum), the highest levels of I-FABP mRNA are encountered in the jejunum (from 10 to 50 pg/μg of total cellular RNA or 200–1000 copies of I-FABP mRNA/cell, ref. 1). A progressive decrease in steady state intracellular levels occurs in enterocytes located from the distal jejunum to terminal ileum [1]. Studies using transgenic mice indicate that cis-acting sequences located between nucleotides -277 and $+28$ of the rat I-FABP gene appear to be necessary for directing its gut-specific, cell-specific and region-specific expression [1]. The precise physiologic function of this abundant [6] cytoplasmic protein is not known. *In vitro* studies using a hydroxyalkoxypropyldextran-based assay described by Glatz and Veer-Kamp [7] revealed that rat I-FABP binds one molecule of long chain (C16–C20) saturated or unsaturated fatty acid in a noncovalent manner with K_d values of 1–4 μM [8]. It's affinity for fatty acids whose chain length is less than 16 carbon atoms is considerably lower [8]. Current hypotheses about the function of I-FABP include a role in the uptake, intracellular targeting and/or metabolic processing of fatty acid within enterocytes. Enterocytes synthesize I-FABP as well as a homologous FABP named liver (L-) FABP because of its initial site of isolation. Gangl and Ockner have shown that luminally-derived fatty acids are largely reesterified in the enterocyte to form triacylglycerol while fatty acids derived from the intestinal blood supply are utilized for energy production and synthesis of phospholipid [9]. It is possible that I- and L-FABP contribute to this metabolic compartmentalization. NMR studies of the interactions of rat I- and L-FABP with ^{13}C-labeled fatty acids are consistent with this hypothesis [10].

Given its small size and unique ligand binding site, we have 'exploited' rat I-FABP as a model for studying the molecular details of fatty acid-protein interaction. In this paper, we describe the steps used to obtain sufficient quantities of the protein for crystallographic studies and the results of our structural analyses.

Expression of rat I-FABP in *E. coli*

It has been difficult to purify I-FABP from the mammalian intestinal epithelium. Separation of I-FABP and L-FABP from one another is arduous and the yields of purified protein modest. Therefore, our approach for obtaining large quantities of I-FABP for structural studies has involved its expression in *E. coli*. To do this, we initially inserted a rat I-FABP cDNA [8] into a prokaryotic expression vector containing the leftward promoter from phage λ (pL). Temperature induction of this promoter is possible if the host strain of *E. coli* contains a thermolabile repressor cI857 which can bind

[1] The primary translation product of rat I-FABP mRNA contains 132 residues [8]. In this and previous reports [22, 24], we have designated the second residue of the primary translation product (Ala) as occupying position 1. This was due to the fact that *E. coli*-derived rat I-FABP lacks its initiator Met. The NH_2-terminus of the protein purified from rat intestinal epithelium is blocked. Indirect evidence suggests that the blocking group may be an acetyl moiety [8]. However, it is not known whether the blocking group is linked to the initiator Met or to the Ala residue.

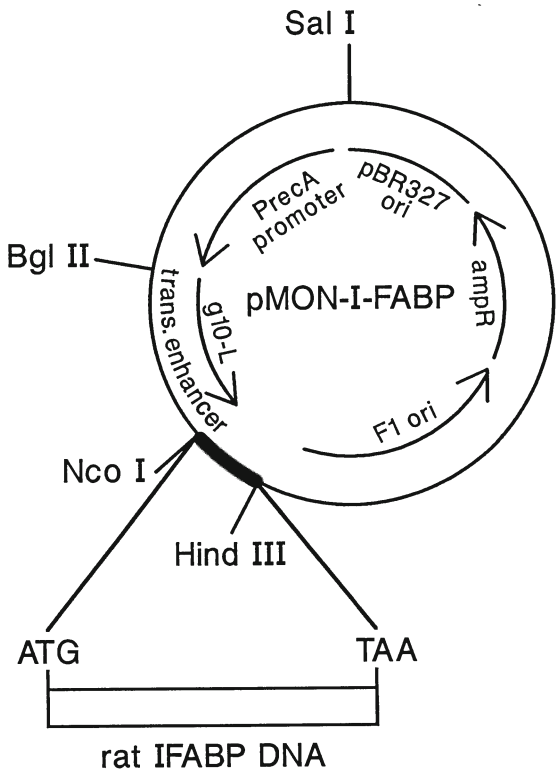

Fig. 1. Schematic drawing of pMON-I-FABP expression vector used to direct synthesis of rat I-FABP in *E. coli.* Rat I-FABP cDNA has been placed just downstream of a translational enhancer element from bacteriophage T7 (5′... AAATAATTTT GTTTAACTTTAAGAAGGAGATATATCC3′). Transcription is regulated by the *recA* promoter (P*recA*) which can be induced by nalidixic acid.

to the promoter at 28° C. Expression of foreign proteins can be achieved in this system by switching the incubation temperature from 28° C to 42° C (see ref. 11). More recently we have used a different system developed by Peter Olins and his colleagues at Monsanto Company (see ref. 12). This pMON vector is shown in Fig. 1. Transcription of I-FABP cDNA contained in the recombinant pMON vector is controlled by a *recA* promoter [13]. There are a number of advantages of using this promoter to direct transcription of I-FABP mRNA. It is 'strong' and can be easily regulated without any temperature restrictions. The *recA* promoter can be readily induced in *E. coli* by adding nalidixic acid to the culture after cells have reached a desired density. In addition, the promoter can be used in

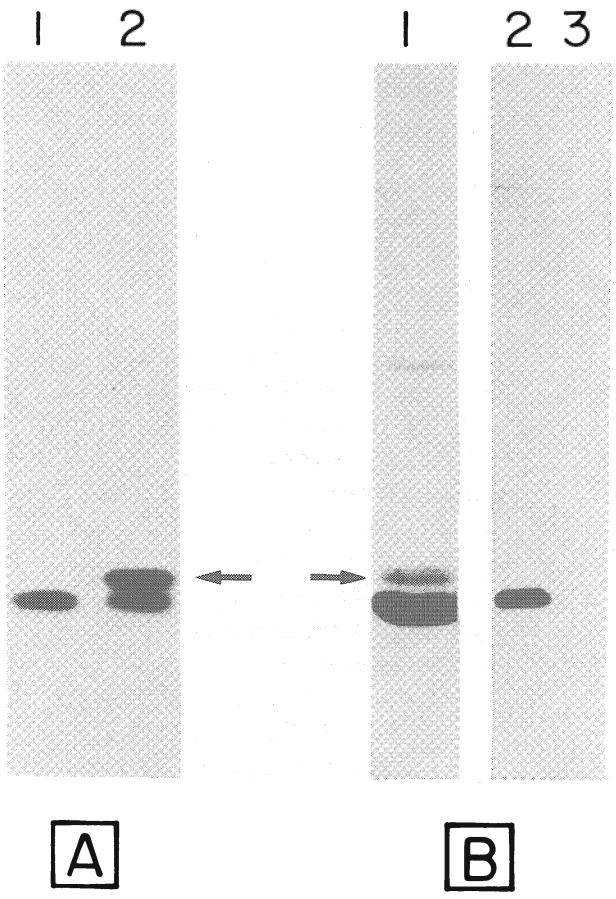

Fig. 2. Readthrough at the stop codon of rat I-FABP mRNA in various strains of *E. coli. Panel A.* A Western blot of an *E. coli* lysate prepared from strain JM101 containing the pMON-I-FABP expression vector. The blot was probed with a rabbit polyclonal, monospecific anti-rat I-FABP serum [8]. Antigen-antibody complexes were visualized using ^{125}I-protein A [45]. An autoradiograph of the blot is shown. *Lane 1* contains a purified rat I-FABP standard (1.5 μg). *Lane 2* represents an *E. coli* lysate (100 μg protein) prepared 1 h after induction of I-FABP synthesis with nalidixic acid. The arrow points to the larger M_r I-FABP species that is derived from readthrough translation at its mRNA's UGA stop codon in this supE$^+$ strain. *Panel B.* Western blots were treated as in panel A with the exception that antibody-antigen complexes were visualized by treatment with a horseradish peroxidase coupled goat anti-rabbit IgG followed by 4-chloro-1-naphthol + H_2O_2 (Tago Immunologicals Co.). *Lane 1* is a lysate (100 μg protein) of *E. coli* strain MG1655 containing pMON-I-FABP. As in panel A, the lysate was prepared 1 h after nalidixic acid induction. Note that in this supE$^-$ strain, considerably less readthrough was observed than in *E. coli* strain JM101. *Lane 2* also represents a lysate (100 μg protein) prepared from strain MG1655. In this case, however, the UGA stop codon in rat I-FABP mRNA was replaced by an inframe UAA stop. No readthrough is detectable. Lane 3 is a lysate of *E. coli* strain MG1655 containing the pMON vector without the I-FABP cDNA. This negative result highlights the specificity of the antibody preparation.

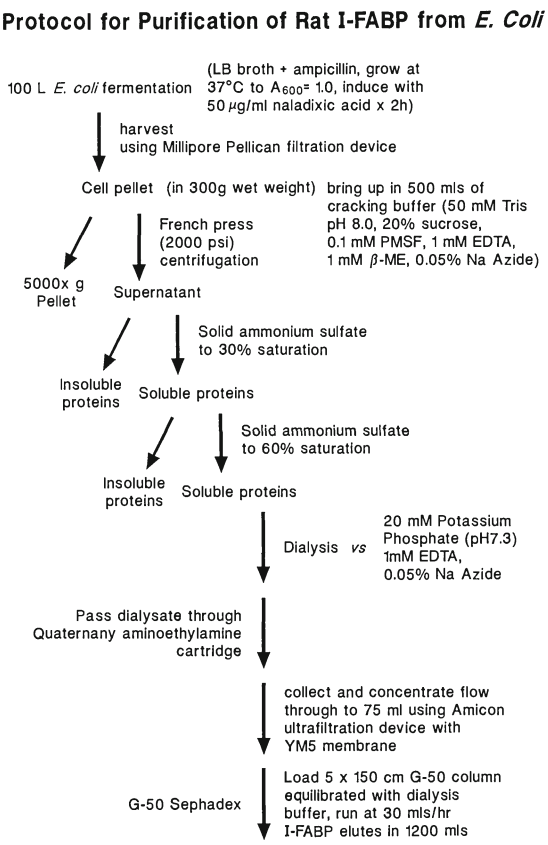

Fig. 3. Protocol used to purify rat I-FABP from *E. coli* containing the pMON-I-FABP expression vector.

most strains of *E. coli* that have a *recA*$^+$ genotype. A ribosome binding site derived from the T7 bacteriophage gene 10 leader (*g10-L*) was interposed between the *recA* promoter and the initiator methionine codon of I-FABP (see Fig. 1). This *g10-L* sequence acts to enhance the efficiency of initiation of translation as well as to increase mRNA stabiltiy [14]. In addition to this translational enhancer element, the pMON vectors contain the origin of single-stranded replication from phage F1 [15, 16]. This feature allows preparation of single-stranded DNA for oligodeoxynucleotide directed mutagenesis or for DNA sequence analysis.

Induction of rat I-FABP synthesis in *E. coli* has no obvious effects on growth kinetics indicating that it is not toxic to the cells. Expression is quite efficient: within 1–2 h after nalidixic induction of the *recA* promoter, the mammalian protein comprises up to 10–15% of the total proteins present in cellular lysates. Many foreign proteins that are expressed in *E. coli* form insoluble aggregates known as inclusion bodies (reviewed in 17). Recovery of active protein from these aggregates is often difficult requiring isolation of inclusion bodies after cell lysis, a denaturation step to solubilize the protein and finally a refolding reaction. This is not the case with I-FABP. It remains quite soluble despite its high steady state levels.

One consequence of the high level of expression of I-FABP in certain strains of *E. coli* is failure to correctly terminate translation at the UAG stop codon present in the rat I-FABP mRNA transcript. The result is a protein with additional COOH-terminal residues. Failure to terminate translation at this codon may be a particular problem when using strains that have supE suppressor tRNA. This suppressor tRNA species has been shown to add a Trp residue to the polypeptide when it encounters the UGA 'stop' [18]. This problem is illustrated in Fig. 2 which shows a Western blot of lysates prepared from several strains of *E. coli* containing our recombinant pMON-I-FABP vector. Strain JM101 is commonly employed for pro-

Table 1. Physical chemical parameters of *E. coli*-derived rat I-FABP

Amino acid residues = 131	
pI = 6.0	
$\varepsilon_{280}^{1\%} = 1.18$	
Space group of crystalline protein =	$P2_1$
Unit cell dimensions (holo-protein)*	a = 36.9 Å, b = 56.8 Å, c = 31,8 Å, β = 114.0°
Unit cell dimensions (apo-protein)*	a = 36.0 Å, b = 56.6 Å, c = 31.6 Å, β = 113,4°

* Although the apo- and holo-unit cells have a similar size, they display a completely different packing arrangement [23].

karyotic expression of foreign proteins. It, however, is supE$^+$. The level of 'readthrough' at I-FABP mRNA's UGA stop in JM101 is very high: nearly 50% of the I-FABP polypeptides contain additional COOH-terminal amino acids (Fig. 2A). NH_2-terminal sequence analysis confirmed that the larger, immunoreactive species denoted by the arrow is in fact I-FABP. Expression of this mammalian protein in MG1655, a supE$^-$ strain of *E. coli,* is associated with considerably less readthrough, even though the overall efficiency of I-FABP synthesis in MG1655 is approximately the same as in JM101 (compare lane 2 in panel A with lane 1 in panel B of the figure).

The three termination codons UAA, UAG, and UGA are recognized by two release factors synthesized in *E. coli.* Release factor 1 (RF1) recognizes UAA and UAG while RF2 recognizes UAA and UGA [19]. There is a very strong bias to use UAA in *E. coli* genes that are expressed at high levels [19]. The selective advantage of UAA could reflect the relatively greater availability of release factors that recognize this stop codon (RF1 and RF2); the other two stop codons (UGA and UAG) are each recognized by only one RF. Since the release factors are generally present in low concentrations [19], they may limit accurate and efficient termination of tranlation in highly expressd foreign transcripts containing UGA and UAG (as is the case for I-FABP mRNA).

We have used site-directed mutagenesis to substitute the naturally occurring UGA codon of rat I-FABP mRNA with an inframe UAA codon (Fig. 1). This eliminates any detectable readthrough when the pMON-I-FABP vector is used in *E. coli* strain MG1655 (compare lanes 2 and 3 in Fig. 2B).

Purification of rat I-FABP from *E. coli*

Purification of rat I-FABP from *E. coli* is remarkably straightforward. An initial ammonium sulfate fractionation is followed by QAE and G-50 chromatography. The details of our purification protocol are presented in Fig. 3. The yield of purified protein generally averages 3–4 mg/L culture using

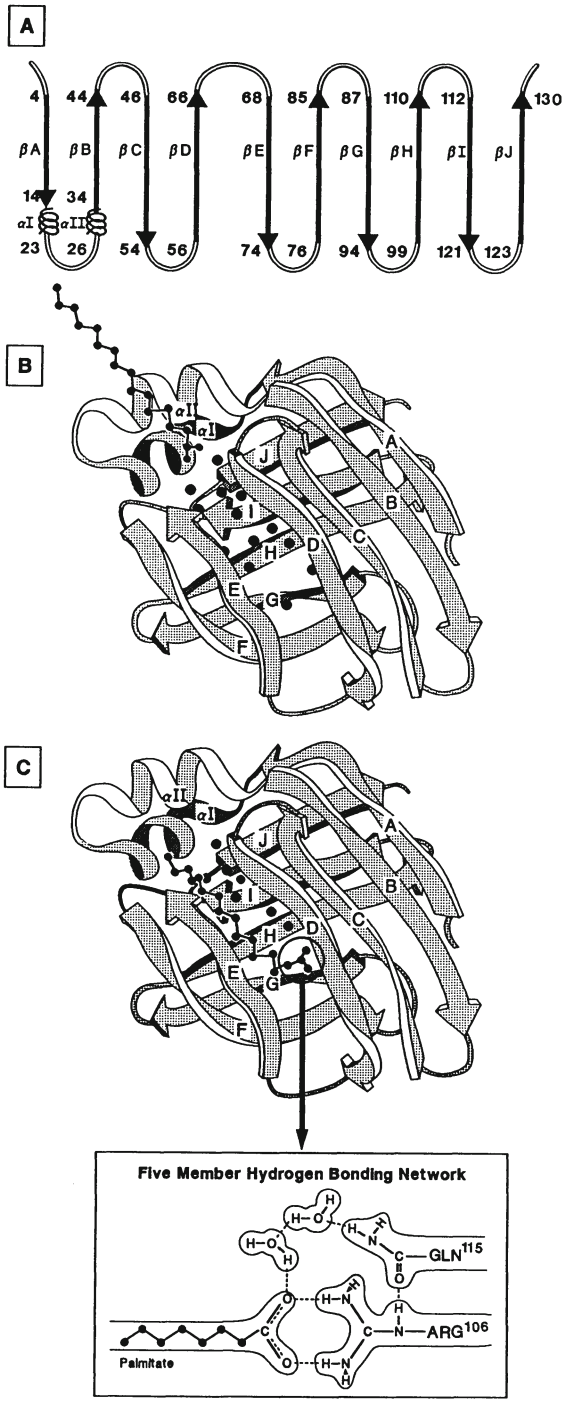

Fig. 4. Schematic views of rat I-FABP. *Panel A* summarized the secondary structural features of crystalline *E. coli*-derived I-FABP. Its 10 β-strands are designated βA-βJ. The two short helical domains are labeled αI and αII. *Panel B* presents a ribbon diagram of apo-I-FABP. Note the 13 ordered solvent molecules [24] located in the core of the β-clam. A proposed entry site for palmitate is indicated. *Panel C* represents the holo-protein with its single molecule of bound palmitate. The inset shows the five-member hydrogen bonding network that includes the carboxylate group of this C16 : 0 fatty acid.

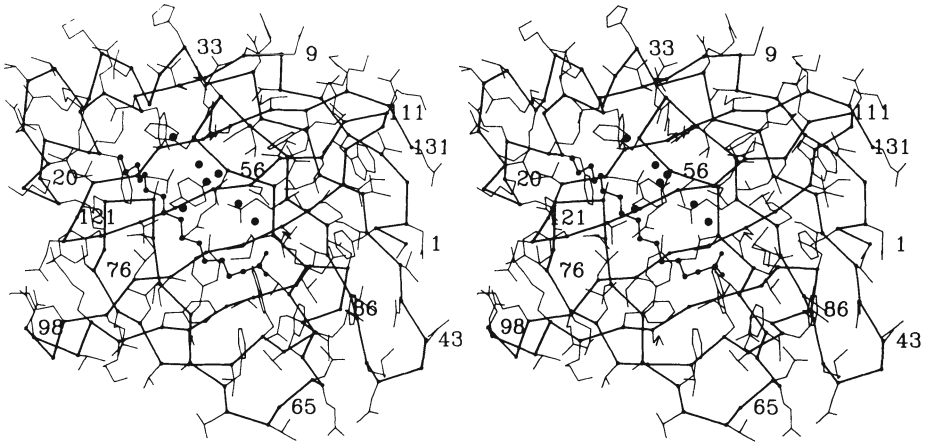

Fig. 5. Stereodiagram of the refined molecular structure of holo-I-FABP showing the location of side chains, ordered internalized solvent (0), and the bound palmitate molecule [22].

the conditions for growth and purification described in the figure.

Synthesis of proteins in *E. coli* is initiated with N-formyl-methionyl tRNA [20]. Formate is removed from essentially all newly initiated proteins. The resulting NH_2-terminal Met is subsequently removed from a large number of endogenous as well as foreign proteins by *E. coli* methionyl aminopeptidases. Automated sequential Edman degradation of purified *E. coli*-derived rat I-FABP indicates that it lacks its initiator Met. This is not surprising since the activity of the aminopeptidase is influenced by the physical-chemical properties of the amino acid which occupies the second position of the primary translation product [21]. In the case of I-FABP this is an Ala. Based on *in vitro* and *in vivo* studies, such an amino acid makes this protein a favorable substrate for the aminopeptidase [21]. Removal of the Met is both efficient and rapid. Pulse labeling studies of *E. coli* strains that express

Table 2. Interactions between palmitate and the side-chains of amino acids located in the core of crystalline *E. coli*-derived rat I-FABP[a]

Carbon atom	Torsional angles (°)	Refined β value	Side-chains and solvent located within 4.5 Å of palmitate
C-1 (COOH)		16.4	Val49, Val60, Phe62, Trp62, Arg106, solvent
C-2	120	19.3	Val60, Tyr70, Trp82, solvent
C-3	286	22.2	Phe68, Tyr70, Trp82, Phe93
C-4	166	20.1	Tyr70, Trp82
C-5	129	27.8	Trp82, Phe93, Ala104
C-6	52	26.5	Leu78, Phe93
C-7	171	24.5	Tyr70, Phe93, Tyr117, solvent
C-8	171	20.8	Leu72, Tyr117
C-9	185	19.9	Tyr117, solvent
C-10	117	48.1	Phe17, Leu72, Tyr117
C-11	299	26.5	Tyr14, Phe17, Tyr117, solvent
C-12	212	32.7	Tyr14, Leu72, 4 solvent molecules
C-13	190	40.4	Tyr14, Met18, Ile23, solvent
C-14	234	31.6	Tyr14, Met18, Ile23, Ala73, solvent
C-15	200	24.7	Ile23, Ala73, Asp74
C-16		51.0	Ile23, Phe55, Ala73, Asp74

[a] Taken from ref. 22.

rat I-FABP indicate that within 1–2 min of induction of synthesis, the initiator Met residue is removed from over half the nascent polypeptide chains [8].

Table 1 lists some of the physical-chemical properties of purified *E. coli*-derived rat I-FABP. Each mol of the purified I-FABP contains approximately one mol of bound fatty acid [8] with palmitate being the most abundantly represented species (~ 0.3–0.5 mol/mol protein, ref. 8). Other bound species include stearate (~ 0.2 mol/mol protein) as well as small amounts of C15 : 0 and C14 : 0.

E. coli-derived rat I-FABP can be readily and efficiently delipidated by passage over a hydroxyalkoxypropyl dextran column (Lipidex 1000, type VI from Sigma) maintained at 37° C [8]. By incubating the apo-protein with a 5-fold molar excess of palmitate and then passing the reaction mixture over the Lipidex column at 4° C, it is possible to remove unbound palmitate and recover I-FABP with a homogeneous population of this bound fatty acid [22].

Table 3. Proteins showing conformational similarities to rat I-FABP

Intracellular lipid binding proteins
Intestinal FABP [22–24]
Liver FABP [45]
Heart FABP [46]
Cellulr retinol binding protein [47]
Cellular retinol binding protein II [48]
Cellular retinoic acid binding protein [49]
Mammary derived growth inhibitor [50]
P2 protein [51]
adipocyte specific aP2 protein [28, 52]
Ileal-specific lipid binding protein (gastrotropin) [53]
α-2-microglobulin family
β-lactoglobulin [41–43]
Serum retinol binding protein [54]
Bilin binding protein (insecticyanin) [55–57]
Bowman's gland (BG) protein from olfactory epithelium (odorant binding protein) [60]
α-1-microglobulin [59]
α-2 μ-globulin [59]
apolipoprotein D [60, 61]
androgen-dependent epididymal secretory protein [62]

References to either the tertiary or primary structure determinations of these proteins are provided in parentheses.

Crystallization of *E. coli*-derived rat I-FABP with bound palmitate

I-FABP-palmitate prepared as described above can be crystallized using the free interface diffusion method [22]. Polyethylene glycol 4000 is used as the precipitant in a solution of 100 mM piperazine-N,N′-bis[2-ethanesulfonic acid] (PIPES) at pH 7.1. The protein crystallizes in the $P2_1$ space group and the unit cell dimensions are a = 36.9 Å, b = 56.8 Å, c = 31.8 Å, with β = 114° (see Table 1). The crystals are extremely resistant to x-ray damage and diffract to greater than 1.8 Å resolution.

The structure of crystalline rat I-FABP with bound palmitate

Multiple isomorphous replacement techniques were used to initially solve the structure of I-FABP to 2.5 Å resolution [23]. The crystal structure of the I-FABP-palmitate complex was subsequently refined to 2.0 Å resolution using a combination of least square methods, energy refinement and molecular dynamics [22]. The combined methods yielded a model with a crystallographic R factor of 17.8%, a root mean square (r.m.s.) bond length deviation of 0.009 Å and a r.m.s. bond angle deviation of 2.85° [22].

A ribbon diagram of the holo-protein is provided in Fig. 4C. A stereoview of the protein indicating the location of all side chains is shown in Fig. 5.

I-FABP contains 10 anti-parallel β-strands in a +1, +1, +1 ... motif (Fig. 4A). The β-strands form two, nearly orthogonal β-sheets: strands βA-βF forms one sheet while βF-βJ form the other (see Figs 4A–C). Together the two β-sheets have the overall shape of a clam shell – leading to the term β-clam [23]. Each strand is composed of 8–10 residues (Fig. 4A) and with the exception of βD and βE, all contain inter-strand hydrogen bonds.

The distance between the Cα chain of βD and βE averages 9 Å, in contrast with the other strands

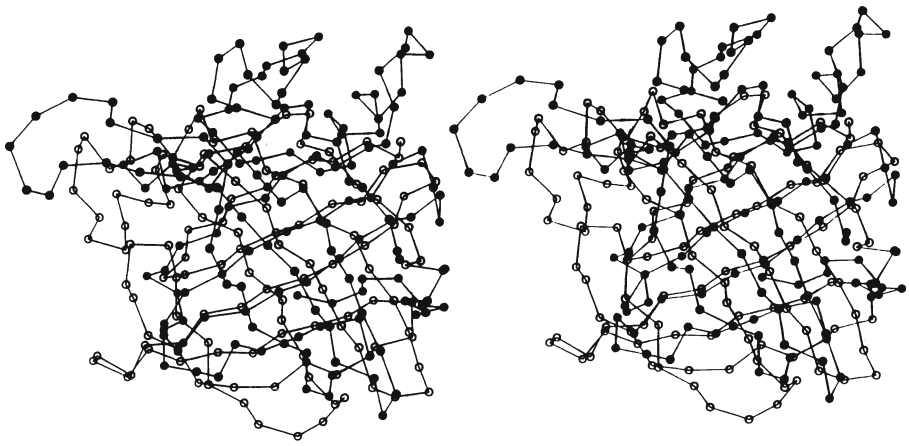

Fig. 6. Comparison of the structures of rat I-FABP and β-lactoglobulin. A Cα chain trace of rat I-FABP (○) and β-lactoglobulin (●) is shown superimposed in stereo.

which are separated by ~3.5 Å. This 'gap' is bounded by βD, βE, the three residue turn between them (Leu[64]-Gly[65]-Val[66]) and two interconnected α helices (αI and αII). αI is an extension of the last part of βA and contains 9 residues (Asn[13] to Met[21]). It terminates with a turn that is formed by Gly[22] and Ile[23]. αII extends from Asn[24] to His[33] and terminates with βB, which spans Asp[34] to Glu[43] (Fig. 4A).

The 'gap' area is actually filled with ordered solvent and the atoms of several side chains. An outer tier of 5 ordered solvent molecules is located on the exterior of the protein midway between βD and βE. Some of these ordered solvent molecules form hydrogen bonds to atoms in the mainchain, producing a network that links βD and βE, filling the place of a 'missing' β-strand. In addition to this outer tier, an inner tier of sidechain atoms is located inside of the strand axis of βD and βE (towards the core of the protein). The side chains of Ile[58], Val[60], Phe[68] and Tyr[70] (Fig. 5) extend into this gap between βD and βE.

E. coli-derived rat I-FABP contains 40 ionizable polar residues out of a total of 131 amino acid residues (Fig. 5). Hydrogen bonds between the side chains of most of these polar residues and ordered solvent molecules create a shell around the protein. The β-clam structure 'allows' several long-range interactions that are stabilized by hydrogen bonds or salt pairs. One such 'long-range' interaction between the carbonyl oxygen of Met[21] (located in the turn between αI and αII) and Arg[95] of βG (Fig. 5). It may aid in stabilizing the two α helices in a position that is close to the β-sheet.

The interior of the β-clam is composed principally of the side chains of hydrophobic amino acids. The aromatic rings of Phe[47], Phe[62] and Phe[68] have similar orientations and appear stacked upon another forming a hydrophobic cluster located near the bottom of the 'gap', close to the carboxylate group of the bound fatty acid (Fig. 5). A second cluster of aromatic side chains that includes Tyr[14], Phe[17], Tyr[117] and Tyr[119] is located near the other end of the methylene chain of the bound palmitate.

The bound fatty acid is located between the two β-sheets with its carboxylate group positioned near the center of the β-clam. Its methylene tail extends in a bent conformation toward αI and αII. The carboxylate group of the fatty acid, two ordered solvent molecules and the side chains of Arg[106] and Gln [115] together form a five-member hydrogen bonding network that is graphically depicted in the insert of Fig. 4C. Note that the ε nitrogen of this buried Arg[106] residue appears in our 2 Å model to be hydrogen bonded to the side chain oxygen of Gln[115]. This 'anchors' the guanidinium group of Arg[106] in an orientation that is planar to the carboxylate group of the C16 : 0 fatty acid (Fig. 5).

The hydrocarbon tail of palmitate bends with a slight left-handed helical twist from the carboxylat-

ed carbon to C-16. The bent hydrocarbon chain sits in a 'cradle' formed by the side chains of aromatic and hydrophobic amino acid residues. Table 2 summarizes these ligand-protein interactions. Seven well-ordered solvent molecules are situated along the concave face of the bent hydrocarbon chain and are hydrogen bonded to the side-chains of polar amino acids residues present in the β-clam. Trp^{82}, Arg^{106}, and a solvent molecule appears to stabilize the major kink in the fatty acid which occurs at C2–3 (Fig. 5 and Table 2). Interestingly, Trp^{82} may be 'bifunctional', contributing both polar and hydrophobic interactions to this portion of the ligand. C5–10 are interposed between, and constrained by, the side chains of Tyr^{117} and Tyr^{70}. The methylene groups spanning the C11–16 positions extend toward the two α helices. The aromatic ring of Phe^{55} appears as a lid covering the omega end of the fatty acid and blocking its access to the protein's surface (Fig. 5).

Comparison of the structures of apo- and holo-I-FABP

To examine the role of the bound fatty acid in maintaining the conformation of rat I-FABP, we have determined the structure of apo-I-FABP to 1.96 Å resolution [24]. The overall structures of the apo- and holo-proteins are very similar: the r.m.s. difference between their Cα atoms is 0.37 Å. The r.m.s. difference between all their main chain atoms is 0.38 Å while the r.m.s. difference between all their side chain atoms is 0.94 Å. However, several important differences between the two structures should be noted. The side-chain orientations of Arg^{56} and Lys^{27} are different in the apo- and holo-proteins. In the holo-protein, the guanidinium group of Arg^{56} points towards βE. One of its amino groups is oriented towards the exterior of the β-clam while the other amino group points towards the interior. The exteriorly oriented amino group is hydrogen bonded to an ordered solvent molecule. The interiorly directed amino group is part of an internal hydrogen bonding network that includes two ordered solvent molecules, Tyr^{70} and Glu^{51}. In the apo-protein, the entire guanidinium group of Arg^{56} is buried in the interior of the protein forming a number of hydrogen bonds with internal ordered solvent and Tyr^{70}, Glu^{51} and Ser^{71}. Differences also exist in the side chain orientation of Lys^{27}. In the apo-protein, its side chain spans a potential solvent accessible opening to the core of the protein. This opening is formed by the tight turns that occur between βC and βD, βE and βF, and αII. The orientation of the side-chain of Lys^{27} in apo-I-FABP closes off the core to external solvent. However, in the holo-protein, the side chain of Lys^{27} is in a position that allows access of exterior solvent to the core of the protein. These differences in the orientations of the side chains of Arg^{56} and Lys^{27} may affect the flow of solvent into and out of I-FABP's interior and play a role in the ligand binding mechanism (see below).

The side chains of residues that provide critical interactions with the bound ligand in holo-I-FABP (c.f. Table 2) are comparably positioned in the core of the apo-protein. However, there are differences in the numbers and locations of ordered solvent molecules. The interior of apo-I-FABP contains 13 ordered solvent molecules. Six of these solvent molecules occupy positions that are also occupied by solvent molecules in the holo-protein (Fig. 5). The apo-protein contains additional solvent molecules that are located at positions close to those occupied by the C1, C2, C8, and C15 atoms of palmitate in the holo-protein. An additional ordered solvent molecule in the apo-protein replaces the carboxylate group of palmitate in the five-membered hydrogen bonding network shown in Fig. 4C.

In summary, these comparisons of the structure of apo- and holo-I-FABP suggest that maintenance of the β-clam structure is not dependent upon the bound fatty acid and that ligand removal has little effect on the orientation of those side chains in the binding pocket that interact with either the carboxylate group, or the hydrocarbon tail of the fatty acid.

A hypothesis about binding mechanisms

Based on our comparisons of the structures of apo-

and holo-I-FABP, we have proposed the following mechanism for binding of palmitate [22, 24]. Entry of palmitate into the interior of the protein may be facilitated by a series of charge interactions between its carboxylate group and charged side chains, for example a basic amino acid located on the protein's surface. This interaction may be followed by entrance of the fatty acid through the solvent accessible opening described above (see Fig. 4B). The carboxylate group would subsequently be incorporated into the five-member hydrogen bonding network and the conformation of the hydrocarbon tail determined by the hydrophobic binding pocket located in the protein's core. Ordered solvent molecules may play an important role in this proposed binding mechanism. The presence of more ordered solvent molecules in the interior of the apo-protein may be an entropic factor which favors fatty acid binding. Solvent flow may be affected by movement of the side chains of Arg^{56} and/or Lys^{27}.

Sequence comparisons among members of the lipocalin superfamily

When the primary structure of I-FABP is used to probe current protein databases with algorithms such as FASTP [25], a number of proteins can be identified with significant amino acid sequence identity. Ten of these proteins are listed in Tabel 3. All are of comparable size. All are cytoplasmic. All appear to bind hydrophobic ligands. Pairwise alignments of their primary structures reveal amino acid sequence identities of ~25–55%. Several of the genes encoding these proteins have also been isolated and sequenced [4, 26–28]. All have four exons. Moreover, intron location is highly conserved among the genes. Together these data are consistent with the notion that I-FABP is a member of a family of intracellular lipid binding proteins that arose from a common ancestral sequence [29].

The tertiary structures of two other family members have been solved: the P2 protein of bovine peripheral nerve myelin [30] and chicken L-FABP[2]. Superimposition of the Cα structures of I-FABP and P2 – two proteins which share 26% identity – revealed r.m.s. differences that were less than 1.6 Å. The remarkable similarity in their secondary and tertiary structures despite their relatively low degree of *primary* sequence similarity suggests that all 10 members of the intracellular lipid binding protein family will exhibit a high degree of conformational 'equivilance'. Both cellular retinol binding protein (CRBP) and cellular retinol binding II have been expressed in *E. coli* [31, 32]. Both proteins have been crystallized [33, 34]. However, the structures of the crystalline proteins have yet to be solved. The diversification in the primary sequences of I-FABP and the cellular retinoid binding proteins is associated with an obvious functional differentiation: for example, I-FABP does not bind all-trans-retinol [32]. The structural basis for this functional differentiation should become obvious once the structures of these family members are solved. Such a solution may be aided by molecular replacement techniques (reviewed in ref. 35) using the coordinates of I-FABP.

McRee and coworkers have recently [36] determined the structure of a 13 kDa prokaryotic protein – the photoactive yellow protein (PYP) from the purple, photoautotrophic bacterium *Ectothiorhodospira halophila*. This structure may provide additional insights about the evolution of this family and about retinoid-CRBP-interactions. The 2.4 Å model reveals a protein with 10 antiparallel β-strands organized into two orthogonal β-sheets. *E. halophila* uses sulfur as an electron sink for photosynthesis [36]. Its chromophore, which resembles retin*al,* undergoes a rearrangement after bleaching by light (most likely a photoisomerization) followed by a relaxation [37]. The photocycle kinetics of this chromophore are similar to those of rhodopsin [37]. The chromophore is located in the interior of this *prokaryotic* 'β-clam'. All of the protein's 15 aromatic and 20 negatively charged side chains form an 'ellipsoidal band' that surrounds the chromophore [36]. McRee *et al.* suggest that this electron rich microenvironment may be very im-

[2] Scapin G, Spadon P, Mammi M, Zanotti G, Monaco HL (Personal communication).

portant for PYP's photocycle [36]. It will be interesting to compare this arrangement with the structures of CRBP or CRBP II with bound retinoid once they are solved.

The structures of the P2 protein of bovine peripheral nerve myelin and *E. coli*-derived rat I-FABP display considerable conformational similarities to members of the α-2-microglobulin family (also known as the lipocalins). Members of this family of extracellular proteins are listed in Table 3. All contain 8 antiparallel β-strands which are organized into two orthogonal β-sheets – forming a so-called β-barrel (see refs. 38, 39, and Table 3). These proteins, like members of the FABP family, bind a variety of hydrophobic ligands which are located within the central cavity of their β-barrels [61].

The conformational similarities between members of the FABP and α-2-microglobulin families were not fully apparent when their primary structures were aligned [40]. Fig. 6 illustrates the nature of these similarities using I-FABP and β-lactoglobulin [41–43] as examples. The superimposed Cα coordinates of each protein reveal that the first three β-strands of I-FABP (βA–βC) align with β-strands A–C of β-lactoglobulin. β-lactoglobulin is 'missing the equivalent of I-FABP's βE and βF. The last (COOH-terminal) four β-strands of each protein are readily superimposable. The area of 'missing' β-strands in β-lactoglobulin opens the protein to solvent – forming a structure analogous to the 'calyx' of a flower. This opening may represent the site of entry/exit for hydrophobic ligands.

Future directions

Further insights concerning the atomic details of I-FABP-fatty acid interactions should be aided by refinement of the model at higher resolution. This should be possible given the relative resistance of the crystalline protein to radiation decay [23] and the fact that current crystals diffract to > 1.5 Å [22, 23]. High resolution neutron diffraction studies should help assess the precise location and nature of ordered solvent molecules in the interior of the holo- and apo-proteins. Site directed mutagenesis of I-FABP, guided by our model of the crystalline protein, should permit direct testing of the role of specific side chains in ligand binding. Given the efficiency of our prokaryotic expression system, large quantities of wild type and mutant proteins may be recoverable for comparative analysis of the functional consequences of site directed mutations. X-ray studies of wild type I-FABP containing fatty acids of differing chain length, steric 'bulk' and stereochemistry should yield additional information about ligand-protein interactions. Studies of other family members will provide, as noted above, information about the structural basis for their functional differences. Ultimately, it *may* be possible to genetically engineer novel intracellular lipid binding proteins which will deliver desired ligands to specific sites within the cell where they may effect the metabolic processing machinery.

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Crystal structure of chicken liver basic fatty acid-binding protein at 2.7 Å resolution

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Key words: fatty acid-binding protein, chicken liver, crystal structure, β-barrel

Abstract

The three-dimensional structure of chicken liver basic fatty acid-binding protein has been determined at 2.7 Å resolution by X-ray crystallography. Phases were calculated using the multiple isomorphous replacement procedure and a preliminary model was built. This model, with an initial R-factor of 0.57, was then improved by a cycle of refinement by simulated annealing which brought the R factor down to 0.32. The protein is structured as a compact 10-stranded-β-barrel which encapsulates a residual electron density that can be interpreted as a fatty acid molecule. The NH_2-terminus portion of the molecule contains two short α-helices. The structure of this liver protein appears very similar to that of the *Escherichia coli* derived rat intestinal FABP recently determined by X-ray diffraction methods.

Introduction

Several proteins that have the property of binding long chain fatty acids have been purified from the cytoplasmic portion of different tissues. They are collectively called fatty acid-binding proteins (FABPs) [1, 2]. Although the precise role *in vivo* of no member of this family has been strictly defined, there is considerable evidence that different FA BPs may serve different functions. The main ones proposed are the intracellular transport and compartmentation of long chain fatty acids, the modulation of the inhibitory effects of fatty acids and their CoA esters on specific enzyme systems and the protection of the cell from detergent effects [3–5]. The term FABP denotes, thus, a functionally loosely defined group of proteins that in common have the property of binding one type of ligand and therefore are likely to be structurally similar or in some way related.

The FABPs characterized so far are small proteins of 127–132 amino acids, reported to have either a single or double ligand binding site for saturated and unsaturated fatty acids [6]. It is this moderate size and simple binding stoichiometry that makes these proteins an attractive system to study fatty acid protein interactions.

Sequences have been determined for rat intestinal (I-FABP [7]), liver (L-FABP [8, 9]) and heart (H-FABP [10]) and human intestine [11], liver [12] and heart FABP [13]. The primary structure of other homologous proteins is also known [14]. Crystals, suitable for X-ray structure determinations, have been reported for bovine liver FABP [15] and rat intestinal FABP [16]. The three-dimensional structure of the latter at 2.5 Å resolution has also been presented [17].

We have recently reported the purification from chicken liver of a FABP that we have called basic fatty acid-binding protein because it has an isoelectric point of 9.0 [18]. We were also able to grow X-ray diffraction quality crystals of this protein that

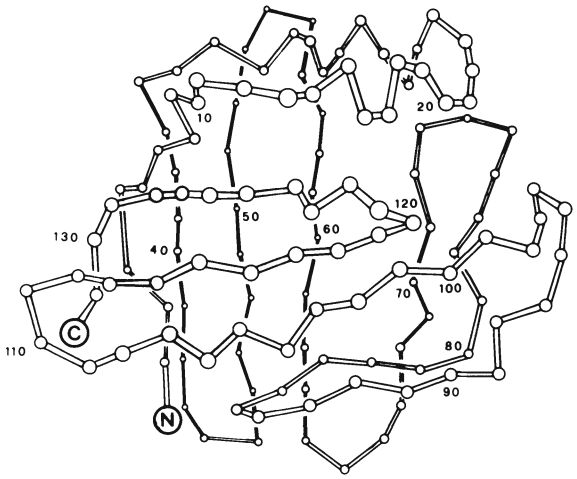

Fig. 1. Alpha carbon chain trace of the molecule. The positions of some overlapping atoms are drawn slightly displaced to facilitate viewing. Notice the perpendicular arrangement of the strands in the two β-sheets.

has a significantly different isoelectric point and amino acid composition from those of the chicken liver FABP recently isolated by Sewell *et al.* [19]. Basic FABP has a molecular weight of 14,000 and is believed to contain one mole of fatty acid bound per mole of protein. Fatty acid analyses after solvent extraction show that most of the endogenous ligand is unsaturated. The physiological role of this molecule is totally unknown, but its amino acid composition shows that it is certainly related to the FABP family of proteins. The primary structure of chicken liver basic FABP has not yet been determined.

In this paper we present the three-dimensional structure of chicken liver basic fatty acid-binding protein determined by X-ray diffraction analysis at 2.7 Å nominal resolution.

Experimental procedures

Purification and crystallization of chicken liver basic FABP have been described previously [18].

All diffraction data were collected to 2.7 Å resolution using the oscillation method and the resulting photographs were processed using standard procedures. An MIR map, calculated with two

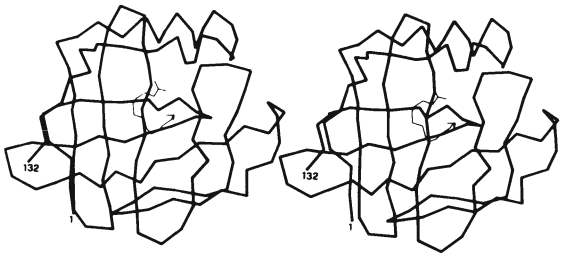

Fig. 2. Stereo diagram of the alpha-carbon chain trace of basic FABP showing the fatty acid model fitted to the extra electron density found in the map. The figure is a photograph from an Evans and Sutherland PS300 screen.

heavy atom derivatives, had a mean figure of merit of 0.63. Inspection of the map showed that it was sufficiently clear to allow tracing of the polypeptide chain. A preliminary model was built that gave a crystallographic R factor of 0.57. After one cycle of refinement using simulated annealing [20], the R factor dropped to 0.32.

A detailed account of the procedure followed to solve this structure will be published elsewhere.

Results and discussion

Since the primary structure of chicken liver basic FABP is not known, the model had to be built using a tentative approximate sequence that was selected making the tacit assumption that the sequence of this protein is homologous to that of other members of the family. Support for this assertion comes from a comparison of the amino acid compositions of the proteins [18]. In more detail, we proceeded in the following way. We started with the primary structure of rat intestinal FABP, one of the two FABPs whose three-dimensional structure is known. When the electron density in the map appeared to fit reasonably well the corresponding amino acid of I-FABP, that amino acid was chosen for the model. If that was not the case, the sequence homology between I-FABP, L-FABP and H-FABP [7, 8, 10] was examined and a different amino acid was selected from another FABP, taking always into account the amino acid composition of the chicken liver protein. In all, 10 amino acids were substituted. Since, in the end, the chosen

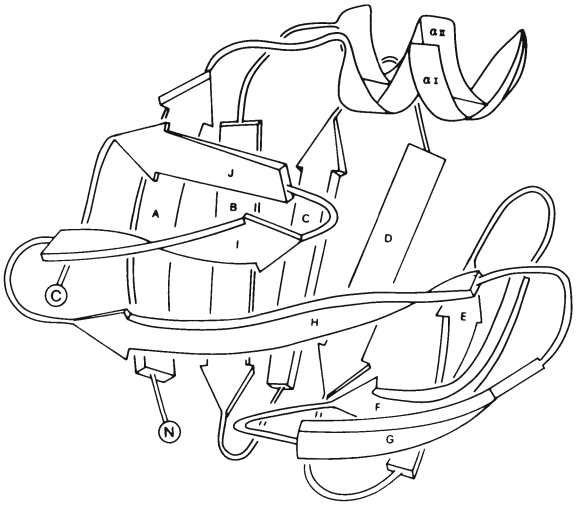

Fig. 3. A cartoon showing the idealized elements of secondary structure.

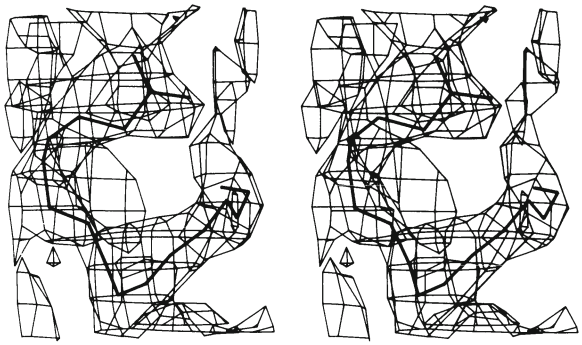

Fig. 4. The electron density interpreted as the bound ligand with the fitted model of an oleic acid molecule superimposed.

sequence does not exactly account for the observed amino acid composition, this sequence must be considered very tentative, and therefore any conclusions regarding particular amino acid interactions must be drawn with great caution. However, the fact that the model built with this primary structure produces a quite acceptable crystallographic R factor, after only one cycle of refinement by simulated annealing, is a good evidence that it is not unreasonable.

Description of the molecule

The basic FABP molecule consists of a single globular domain of about 40 ⋆ 23 ⋆ 30 Å, composed of 10 strands of anti-parallel β-sheet and two short α-helices. Figure 1 shows the α-carbon chain trace of the molecule, Fig. 2 is a stereo diagram and Fig. 3 is a cartoon showing the idealized elements of secondary structure. The 10 strands of β-structure, labelled following the notation used by Sacchettini *et al.* [17] for the structurally very similar rat intestinal FABP run from residues 5–10 (strand A), 37–42 (strand B), 47–52 (strand C), 57–62 (strand D), 68–72 (strand E), 76–83 (strand F), 88–93 (strand G), 99–108 (strand H), 113–118 (strand I) and 123–130 (strand J). The two α-helices run from residues 14–21 (α-I) and 23–32 (α-II). These assignments are to be considered tentative, because some of the amino acids in the model will have to be changed when the protein sequence is known and because the resolution of this electron density map is modest. A future revision with better resolution data after the primary structure of the molecule is known may change them slightly.

The 10 strands of β-structure are organized into two orthogonal β-sheets: the first includes strands A, B, C, D, E and F, the second strands F, G, H, I and J. Strands F and J are in contact with both sheets, this being made possible by sharp changes in direction that occur in our current model in positions 80 and 128. The two α-helices I and II are inserted in between strands A and B and have their axes approximately parallel to strands G, H, I and J. Although the hydrogen bond pattern in the beta sheets has not yet been completely determined, it is likely that it may present irregularities. Some alpha carbon – alpha carbon distances appear in this model to be somewhat larger than expected, particularly between strands D and E where they can reach a maximum value of about 9 Å.

The point through which the ligand can enter or exit its binding site is not obvious in the model and there is at present no structural information whatsoever on any apo-FABP from which it could be inferred.

Not surprisingly, the overall architecture of this chicken liver FABP appears very similar to that of rat intestinal FABP [17] and also to that of bovine P2 myelin protein [21]. These three proteins are

thus seen to belong to the same structural family, which is likely to include many other members as suggested by the many sequence homologies already found.

Fatty acid binding

While building the protein molecule model, it became apparent that there was in the map a very clear electron density, not connected to the polypeptide chain, that could be interpreted as a fatty acid molecule. Figure 4 shows the appearance of this electron density in the map. To this electron density we have fitted an oleic acid molecule, because the cis-bond in position 9–10 gives us a good fit to the density and because we know from fatty acid analysis that oleic acid is one of the ligands bound to the protein in our preparation [18]. In the model we have chosen to place the carboxyl group facing residue 29 and the hydrocarbon tail close to residues 8, 60, 70, 71, 72, 78, 83 and 103.

Since the sequence of basic FABP is not known, we do not know the identity of these residues, in particular of residue 29, but an examination of sequence homologies shows that residue 29 is a highly conserved arginine. The electron density we observe close to the position of this residue is compatible with the presence of an arginine in basic FABP.

If our interpretation, based only on the characteristics of the electron density observed and subject to the caveats we have mentioned above, is correct, the binding of the ligand would be, in this case, different from that observed in the case of the two other FABPs whose three-dimensional structure is known. In rat intestinal FABP the carboxyl group of the ligand has been placed close to Arg 127 [17], whereas in the P2 myelin protein the residues believed to be involved in the carboxyl binding are Arg 106 and 126 [21]. Higher resolution data and sequence information, the two prerequisites to a more accurate refinement of the model, are essential to either confirm or disprove our hypothesis which, if corroborated, would suggest alternative binding modes of analogous ligands to highly similar macromolecules.

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^{13}C NMR studies of fatty acid-protein interactions: comparison of homologous fatty acid-binding proteins produced in the intestinal epithelium

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Summary

A high-resolution, solution-state NMR method for characterizing and comparing the interactions between carboxyl ^{13}C-enriched fatty acids (FA) and individual binding sites on proteins has been developed. The utility of this method results from the high degree of resolution of carboxyl from other carbon resonances and the high sensitivity of FA carboxyl chemical shifts to intermolecular environmental factors such as degree of hydrogen-bonding or hydration, degree of ionization (pH), and proximity to positively-charged or aromatic side-chain moieties in proteins. Information can be obtained regarding binding heterogeneity (structural as well as thermodynamic), binding stoichiometries, relative binding affinities, the ionization behavior of bound FA and protein side-chain moieties, the physical and ionization states of unbound FA, and the exchange rates of FA between protein binding sites and between protein and non-protein acceptors of FA, such as model membranes.

Cytosolic fatty acid binding proteins represent an excellent model system for studying and comparing fatty acid-protein interactions. Prokaryotic expression vectors have been used to direct efficient synthesis of several mammalian intestinal FABPs in *E. coli*. This has enabled us to isolate gram-quantities of purified FABPs, to introduce NMR-observable isotopes, and to generate FABP mutants.

The intestine is the only tissue known to contain abundant quantities of more than one FABP homologue in a single cell type. It is likely that these homologous FABPs serve distinct functional roles in intestinal lipid transport. This paper presents comparative ^{13}C NMR results for FA interactions with FABP homologues from intestine, and the functional implications of these analyses are discussed.

Abbreviations: FA – Fatty Acid(s), FABP – Fatty Acid Binding Protein(s), I-$FABP_c$ – Cytosolic rat intestinal Fatty Acid Binding Protein, L-$FABP_c$ – Cytosolic rat liver Fatty Acid Binding Protein, CD – Circular Dichroic spectroscopy

The fatty acid binding proteins (FABPs) belong to a 10-member family of cytosolic proteins that bind amphiphilic ligands. In addition to intestinal, liver, and heart FABPs, this family includes three distinct cellular retinoid binding proteins, the myelin P2 and adipocyte P2 proteins, mammary-derived growth inhibitor, and gastrotropin [1–3]. The individual functional roles of FABP homologues are unknown, although they are thought to function in some manner in the uptake, transport, and/or trafficking of amphiphilic lipids. Different members of the family may serve similar functions in different

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tissues, or alternatively, distinct functions in the same tissue.

Only one cell type, the absorptive enterocyte in the small intestine, is known to express more than one FABP. Hence, the FABPs from intestine (the so-called intestinal FABP, liver FABP, and gastrotropin) represent unique model systems for correlating structural diversification with possible functional diversification within this protein family. Gastrotropin, once thought to be a hormonal stimulant of gastric acid secretion, was recently found to share considerable sequence identity with liver FABP (37%) and intestinal FABP (24%; ref. [3, 4]). Unlike intestinal and liver FABPs, which are relatively more abundant in the proximal jejunum, gastrotropin synthesis is limited to the distal ileum [5]. Since bile salts are largely absorbed in the distal ileum as part of the enterohepatic circulation, gastrotropin may represent the heretofore postulated intracellular bile salt transport protein.

One approach for elucidating the functional roles of these binding proteins is to compare their structural interactions with ligands using NMR [6–9]. This NMR approach has a number of advantages over other biochemical and spectroscopic binding assays. First, binding can be monitored and quantitated without need for separating bound from free ligand. Therefore, equilibrium binding conditions need not be perturbed. Second, the ^{13}C nucleus represents an essentially nonperturbing probe; ambiguities encountered with structure-perturbing spin-labelled or fluorescent-labelled fatty acids can then be avoided. Third, the FABP preparation used for NMR binding studies need not be delipidated; effective delipidation of FABP preparations (especially rat L-FABP) can result in protein aggregation and precipitation and possible disruption of the native protein conformation. Fourth, additional information is obtained from the NMR spectrum, such as the visualization of distinct binding environments on proteins (binding site structural heterogeneity), the relative occupation and filling of individual binding sites (thermodynamic heterogeneity), the ionization states of bound FA molecules, and the presence or absence of FA-protein electrostatic interactions in individual sites. Fifth, dynamic information regarding the motions of bound ligands and the exchange rates of FA between binding sites and between bound and unbound pools can be obtained. Finally, the ionization and physical states of unbound FA in the aqueous medium can be monitored.

A limitation of this method is that large amounts of pure protein are required: ~ 35 mg of FABP per NMR sample. However, advantage can now be taken of efficient prokaryotic expression systems. The cDNAs for rat I-FABP$_c$, L-FABP$_c$, and porcine gastrotropin have been cloned [3, 10, 11], introduced into prokaryotic expression vectors [12, 13], and milligram to gram quantities of their primary translation products subsequently purified from *E. coli*.

The purpose of this presentation is to demonstrate how ^{13}C NMR spectroscopy, combined with recombinant DNA techniques, can be used to directly compare the binding properties of different FABPs from intestine *in vitro*. The NMR results highlight differences between I- and L-FABP$_c$ with regard to the number of bound FA, the environments and locations of bound FA carboxylate groups, and the pH sensitivity to FA dissociation and conformational changes. In turn, these structural differences provide insights into possible functional roles of FABPs in intestinal lipid absorption and transport *in vivo* [14].

Materials and methods

The procedures for sample preparation and characterization, as well as ^{13}C NMR data collection and analysis, have been described in detail elsewhere [14].

Results

The complete natural abundance ^{13}C spectrum of *E. coli*-derived rat I-FABP$_c$, without added ^{13}C-enriched fatty acid, is shown in Fig. 1. Several general regions can be defined: methyl and methylene carbons (~ 10–45 ppm), alpha carbons (45–75 ppm), aromatic carbons (115–140 ppm), and carbonyl and carboxyl carbons (165–185 ppm).

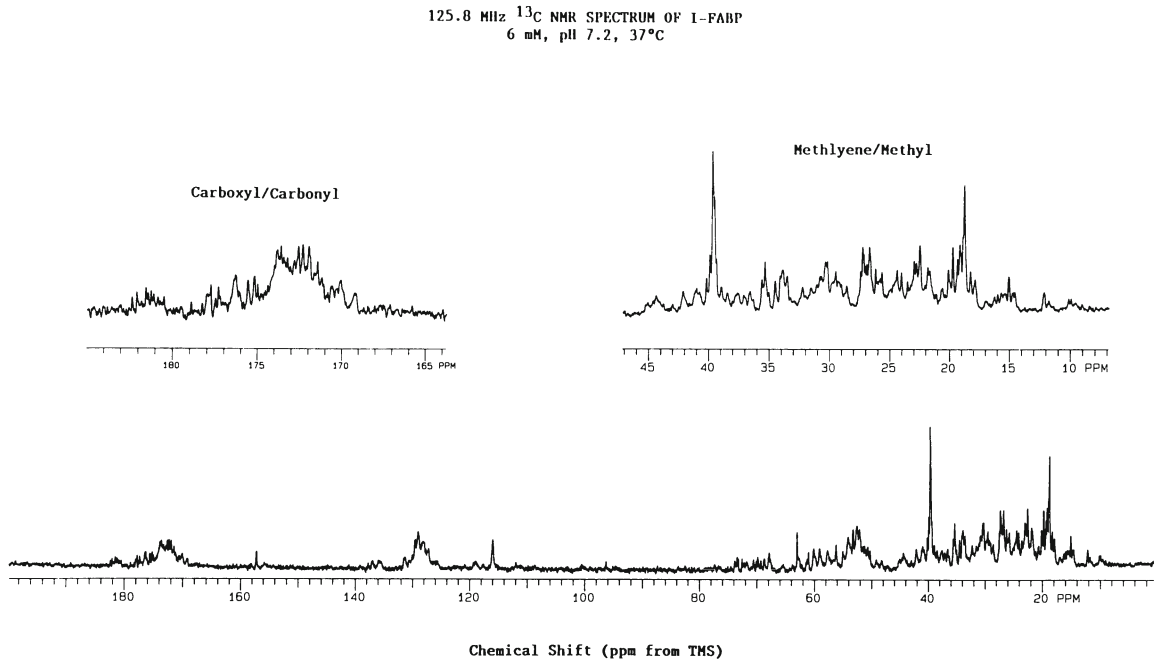

Fig. 1. Natural abundance 125.8 MHz proton-decoupled carbon-13 NMR spectrum of rat I-FABP$_c$ derived from *E. coli*, at 6 mM, pH 7.2, 37° C. TMS, tetramethylsilane.

Expansions of the carboxyl/carbonyl regions and methylene/methyl regions are displayed in the insets to Fig. 1. The narrow intense resonance at 39.52 ppm represents the ε-carbons of Lys residues and is often used as an internal chemical shift reference (after calibration against external tetramethylsilane).

In the spectra for FA.FABP complexes shown below, attention is focused primarily on the carboxyl/carbonyl region, since resonances for carboxyl ^{13}C-enriched fatty acids appear in this region. The rationale for using carboxyl ^{13}C enrichment is twofold. First, FA carboxyl resonances are completely resolved from natural abundance protein resonances (except glutamate carboxyls). Second, FA carboxyl resonances are extremely sensitive to intermolecular factors such as hydrogen-bonding or hydration, degree of ionization (pH), involvement in charge-charge interactions, and proximity to ring currents [6–9].

To directly compare the maximum binding stoichiometries and relative affinities of I- and L-FABP$_c$, a sample containing *both* I-FABP and L-FABP was titrated with ^{13}C-enriched palmitate and NMR spectra were accumulated at selected mole ratio increments. The results are shown in Fig. 2. The sample temperature was maintained at 26° C to ensure that any unbound palmitate would form a crystalline phase, rather than a liquid crystalline (bilayer) phase [14, 15], since the latter may compete with FABP for FA binding. Carboxyl peaks were observed at 182.2 ppm (peak L), 181.4 ppm (peak I), and 181.0 ppm (peak g). Peak L corresponded to palmitate bound to L-FABP$_c$, and peak I, palmitate bound to I-FABP$_c$ [14]. Peak g represents natural abundance glutamate carboxyl carbons from both proteins. The observation of two narrow distinct peaks corresponding to FA bound to each protein indicated that the exchange rate of palmitate between L-FABP$_c$ and I-FABP$_c$ was slow on the NMR chemical shift time scale. An upper limit for the exchange rate is $< 40\,\text{sec}^{-1}$ with a lifetime > 25 msec. At 2/1/1 mole ratio (Fig. 2A), the relative intensities of peaks L and I were similar. However, with increasing FA/FABP mole ratio, the intensity of peak L increased to a greater

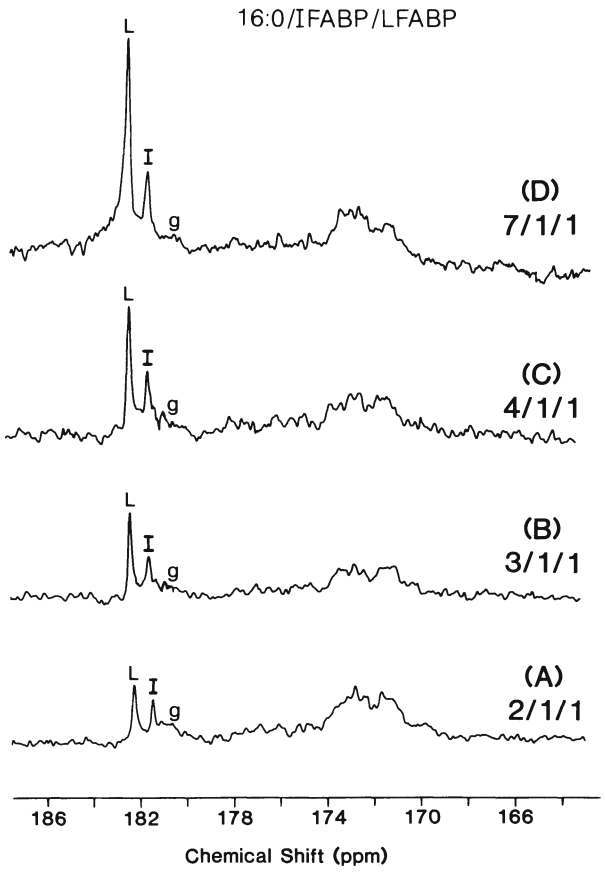

Fig. 2. Carboxyl/carbonyl region of proton-decoupled ^{13}C NMR spectra of complexes of carboxyl ^{13}C-enriched palmitate with *E. coli*-expressed I-FABP$_c$ and L-FABP$_c$, all at pH 7.2 and 26° C. The NMR sample contained approximately equimolar amounts of I- and L-FABP$_c$ (0.76 mM and 1.1 mM, respectively) and increasing amounts of carboxyl ^{13}C-enriched palmitate. The approximate mole ratio of total FA to I- to L-FABP$_c$ in the sample is noted above the right edge of each spectrum. Peaks L, I, and g are described in the text. The NMR internal sample temperature was 26° C. Number of spectral accumulations: 18,283 (A); 6,057 (B); 4,000 (C); 12,000 (D); 5 Hz linebroadening was used in spectral processing. Reproduced from Journal of Biological Chemistry [14]; used by permission.

extent than did peak I (Fig. 2B-D). Spectra at 6/1/1, 8/1/1, and 10/1/1 mole ratio values (now shown) were essentially identical to that at 7/1/1 mole ratio (Fig. 2D) and indicated that both proteins had become saturated with palmitate. Although unbound FA was not apparent in the NMR spectra shown in Fig. 2, the samples contained suspended 1 : 1 acid-soap crystals [14–16].

Partition ratios, defined as the mole quantities of FA associated with L-FABP$_c$ divided by that of I-FABP$_c$, were determined from spectra shown in Fig. 2. In Fig. 2A, the partition ratio was 1.4, indicating that L-FABP$_c$ had an affinity for the first mole of bound palmitate approximately equal to that of I-FABP$_c$. This NMR result is in close agreement with the relative affinities as determined by a Lipidex binding assay [13]. In Fig. 2D, the partition ratio was 2.9, indicating that L-FABP$_c$ bound approximately 3 times more palmitate than did I-FABP when both proteins were saturated with ligand. Since I-FABP$_c$ only binds one mole of FA per mole of protein [13, 14], the maximum FA binding stoichiometry of L-FABP$_c$ in the absence of a competing bilayer phase was 3 moles of FA per mol protein.

To directly compare the ionization behavior and dissociation of FA from I-FABP and L-FABP as a function of pH, an NMR sample containing both proteins and saturating amounts of ^{13}C-enriched palmitate, similar to that in Fig. 2D, was titrated with 1N HCl or KOH. Spectra were accumulated at different pH values as shown in Fig. 3. At pH 8.9, the NMR spectrum was essentially identical to that at pH 7.4. However, at pH values < 7.2, the *intensity* (area) of peak L decreased relative to that of peak I (Fig. 3b-g) and peak L nearly disappeared from the spectrum below pH 5. In addition, the *chemical shift* values for peak L, but not peak I, decreased with decreasing pH. When the pH was 'reversed' from 4.9 (Fig. 3g) to 7.2 (Fig. 3h), the chemical shift values for peak L returned to their original values at pH 7.2, but the intensity of peak L was less than its original value (compare panels b and h). However, when the pH values were kept above 5.1, the NMR spectra were completely reversible. These results indicated that FA dissociated reversibly from L-FABP, but not I-FABP, at and below physiological pH.

The ionization behavior of FA bound to I-FABP and L-FABP was monitored by the changes in chemical shifts of peaks I and L as a function of pH. The chemical shift values for peak I as a function of pH are shown in Fig. 4, right lower panel. No

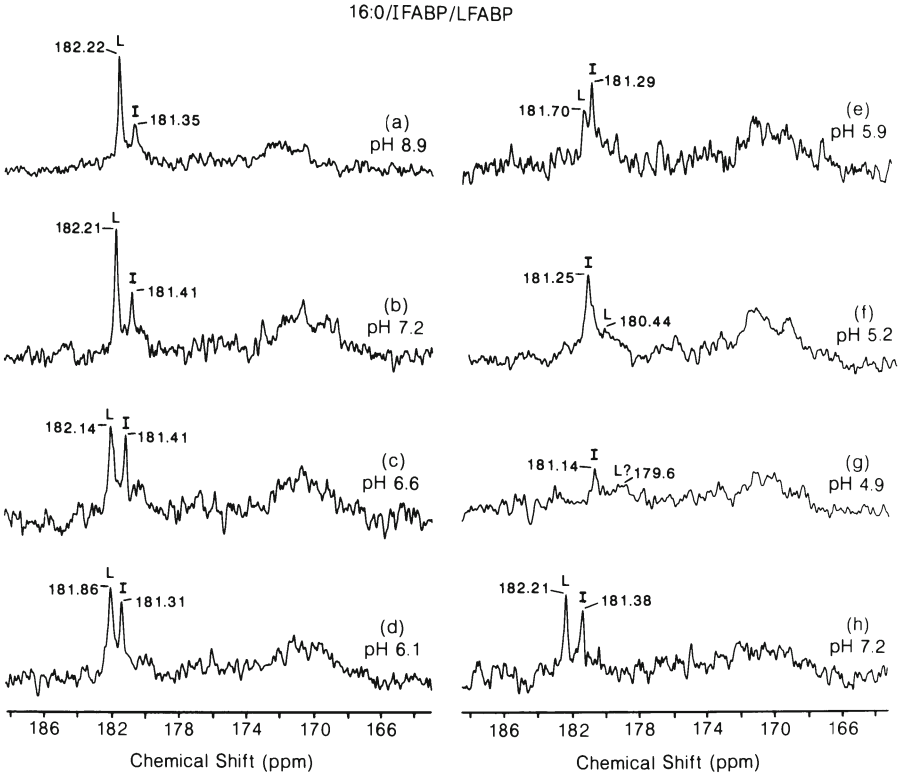

Fig. 3. Carboxyl/carbonyl region of proton-decoupled ^{13}C NMR spectra of complexes of carboxyl ^{13}C-enriched palmitate with *E. coli*-expressed I- and L-$FABP_c$ at different pH values, all at 26° C. A single sample containing palmitate, I-, and L-$FABP_c$ (8 : 1 : 1 sample mole ratio) was titrated with 1 N HCl from pH 8.9 to 4.9 (panels a–g) and then reversed back to pH 7.2 with 1 N KOH (panel h). Reproduced from the Journal of Biological Chemistry [14]; used by permission.

change in chemical shift was observed between pH 5.0 and 11.0, and only a slight decrease occurred below pH 5.0. In contrast, the chemical shift values for peak L decreased with decreasing pH below pH 7.0 in a manner consistent with protonation of the FA carboxyl group, which was fully ionized (anionic) at neutral pH. Its estimated apparent pK_a value (assuming a complete ionization shift of 4.8 ppm for FA), was approximately 4.7–4.8. This value agreed with the pK_a values obtained for monomeric FA in water in the absence of protein [7].

The ionization behavior of FA bound to I- and L-$FABP_c$ suggested that the FA carboxylate moieties were solvent-inaccessible and -accessible, respectively. For I-FABP, it was likely that the FA carboxylate group was involved in an ion-pair electrostatic interaction with a cationic residue on I-FABP.

To determine whether the pH-dependent changes in FA dissociation from L-$FABP_c$ were accompanied by conformational changes in L-FABP, as defined by changes in circular dichroism molar ellipticities, individual samples of palmitate/L-FABP and palmitate/I-FABP complexes were titrated with acid or base and CD spectra were recorded as a function of pH. The molar ellipticities at 217 nm for L-FABP and I-FABP, and the NMR intensities of peaks I and L derived from the NMR spectra in Fig. 3, are plotted as a function of pH in Fig. 4, left panels. The molar ellipticities for L-FABP decreased with decreasing pH below pH 7.5 (lower left panel, filled triangles). Concurrently, the NMR intensity of peak L decreased with decreasing pH (upper left panel, filled triangles). In con-

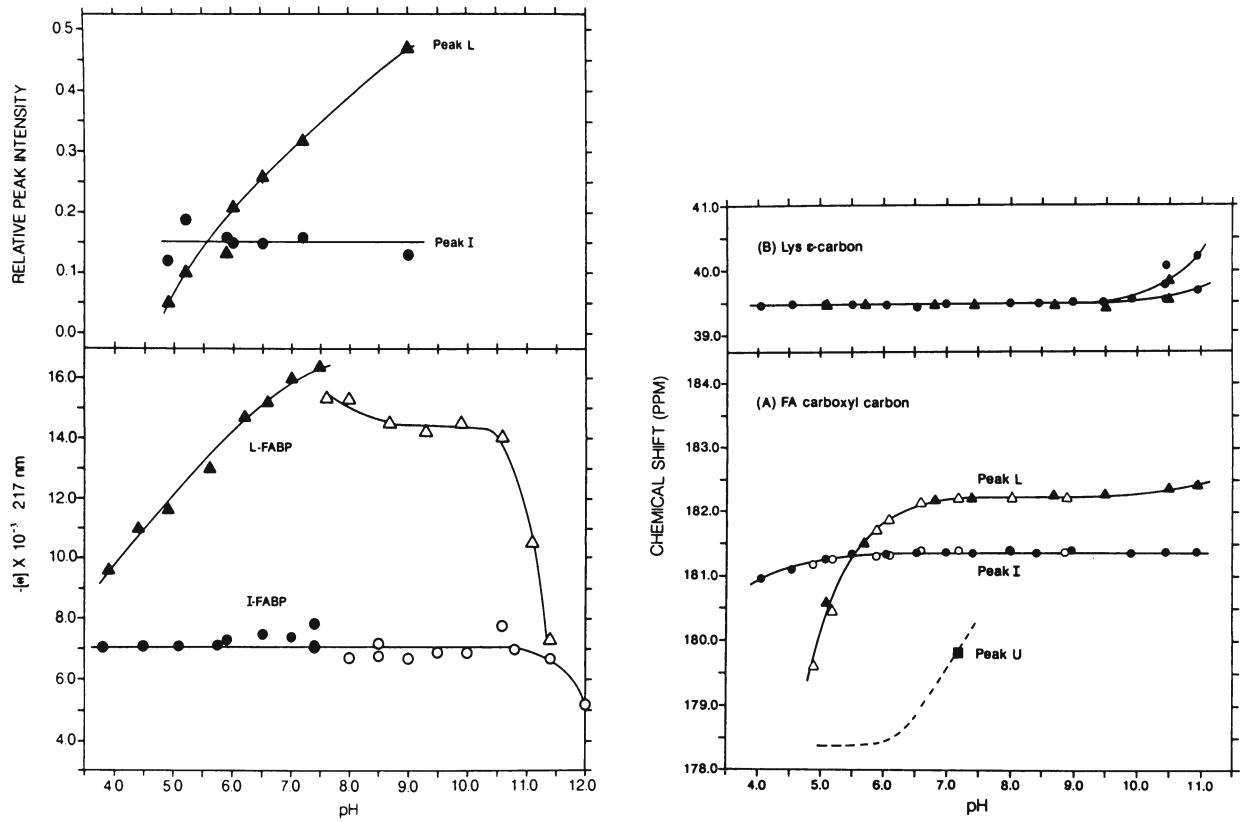

Fig. 4. NMR relative peak intensities and CD molar ellipticites as a function of pH (left panels) and NMR chemical shifts as a function of pH (right panels). In the upper left panel, the relative intensities of NMR peaks L (filled triangles) and I (filled circles) were derived from spectra in Fig. 3. In the lower panel, molar ellipticites at 217 nm were derived from circular dichroic spectra for FA/I-FABP$_c$ (circles) and FA/L-FABP$_c$ complexes (triangles), both at 1/1 FA/protein mole ratio. The filled symbols represent samples titrated from neutral to low pH with HCl, and the open symbols, samples titrated from neutral to high pH with KOH. All experiments were carried out at 26° C. NMR titration curves for FA carboxyl carbons (lower right panel) and protein Lys ε-carbons (upper right panel) are shown. The filled circles and filled triangles represent samples containing ^{13}C-enriched palmitate and I- or L-FABP$_c$, respectively, and the open circles and open triangles represent a single sample containing palmitate and both I- and L-FABP$_c$, corresponding to the sample in Fig. 2D. The dashed line represents an NMR titration curve for unbound bilayer phase oleate in a sample containing no protein [16, 18]. Chemical shift values for palmitate/I-FABP$_c$ samples at pH 11.8 (not shown) were 181.35 for peak I and 40.6 and ~ 40.0 ppm for Lys ε-carbon peaks. Reproduced from the Journal of Biological Chemistry [14]; used by permission.

trast, the molar ellipticities of I-FABP (lower left panel, circles) and the NMR intensity of peak I (upper left panel, circles) were essentially unaffected by pH.

Discussion

The molecular interactions between carboxyl ^{13}C-enriched palmitate and *E. coli*-derived rat intestinal and liver FABP$_c$ in solution have been directly compared using high-resolution ^{13}C NMR spectroscopy. A shortcoming of the ^{13}C NMR binding assay is that large quantities of pure protein are required. In the studies described here, advantage was taken of efficient *E. coli* expression systems for rat I- and L-FABP$_c$ [12, 13] to obtain sufficient quantities of protein for NMR studies. In addition, isolation of rat I- and L-FABP$_c$ from bacterial cells ensured that both proteins were exposed to a similar, non-

complex ligand environment *in vivo* [17], thus, allowing a more direct comparison of the binding properties of the isolated proteins *in vitro*.

I-FABP$_c$ and L-FABP$_c$: Direct Comparison of their binding properties

The structural features of FA-protein interactions for I-FABP$_c$ and L-FABP$_c$ are summarized in Table 1. Rat I- and L-FABP$_c$ exhibited differences in FA binding stoichiometries at neutral pH. I-FABP$_c$ bound a maximum of 1 mol of palmitate per mol protein under all conditions employed. L-FABP$_c$ bound up to 3 moles of palmitate, depending on whether unbound FA formed a crystalline or liquid crystalline phase. The observed variability in binding stoichiometry for L-FABP$_c$ may be related to variability in the partitioning of FA between second or third binding sites on L-FABP$_c$ and unbound phases [18]. I-FABP$_c$ exhibited no such variability and appeared to contain a single, high affinity FA binding site.

Although L-FABP$_c$ bound more than one mole of FA per mole protein, FA carboxyl carbon peak heterogeneity was not observed. This observation is in sharp contrast to ^{13}C NMR spectra for FA-albumin complexes, where at least 5 distinct carboxyl resonances, corresponding to FA bound to distinct binding sites, were observed. For albumin, one of these resonances (peak c; see ref. [6, 7]) represented more than one mole of bound FA and exhibited a chemical shift value and ionization behavior essentially identical to that of peak L in this study. As observed with albumin, two or more molecules of FA bound to distinct sites on L-FABP could experience a similar chemical environment and hence, exhibit identical chemical shift values. Carboxyl carbon peak heterogeneity was also not observed for FA/*I*-FABP$_c$ complexes, but this finding is not unexpected for a protein that contains only one FA binding site.

The second major difference observed between I- and L-FABP$_c$ was in the general locations of their bound FA carboxyl(ate) groups. At neutral pH, the carboxyl chemical shift value for FA bound to I-FABP$_c$ was lower than the value observed for FA bound to L-FABP$_c$ (181.4 ppm vs. 182.2 ppm, respectively). In the absence of other contributing factors, exposure of FA carboxyl(ate) moieties to solvent water molecules results in FA-water hydrogen bonding, 'delocalization' of electrons from the carboxyl carbon (deshielding), and an increase in the observed chemical shift value. Therefore, the lower carboxyl chemical shift value observed for FA bound to I-FABP was generally suggestive of a

Table 1. Comparison of FA-protein interactions for I-FABP and L-FABP

Features	I-FABP	L-FABP
FA binding stoichiometry	1	2 or 3[a]
FA carboxyl chemical shift	181.4 ppm	182.2 ppm
FA carboxyl peak heterogeneity	not detected	not detected
FA ionization behavior	$pK_{app} << 4$[b]	pK_{app} 4.7–4.8
FA carboxylate binding location	solv.-*in*accessible; interior	solv.-accessible; near surface
FA-protein ion-pair electrostatic interactions	detected (Arg^{127}/Arg^{106})[c]	not detected
FA dissociation from protein	pH-*in*sensitive	pH-sensitive
Conformational changes[d] near neutral pH	pH-*in*sensitive	pH-sensitive
Relative binding affinity[e]	same as L-FABP	same as I-FABP

[a] The binding stoichiometry of L-FABP depends upon the physical state of the abound FA and the sample pH (see Discussion). In the presence and absence of a competing bilayer phase, up to 2 and 3 moles, respectively, of palmitate FA bound to L-FABP at pH 7.2.
[b] FA bound to I-FABP exhibited little or no change in ionization state between pH 7.0 and 4.0. Abbreviation: pK_{app}, apparent pK_a value.
[c] Based on current interpretation of 2.0 Å electron density map of crystalline I-FABP [19].
[d] As defined by changes in CD molar ellipticities at 217 nm.
[e] Relative binding affinity for the *first* mole of FA per mole FABP. After ref. [14]. Used by permission.

binding environment less solvent-accessible than that for L-FABP.

Differences in solvent accessibility were further assessed by comparing the ionization behavior of bound FA molecules. FA bound to I-FABP exhibited little or no ionization shift between pH 7.0 and 4.0. In contrast, FA bound to L-$FABP_c$ exhibited an ionization shift and an apparent pK_a value characteristic for solvent-accessible FA carboxylate moieties. Therefore, the carboxylate group of FA bound to I-$FABP_c$ appears to be located in a more interior region of the protein and probably involved in ion-pair electrostatic interactions, whereas the carboxylate groups of FA bound to L-FABP appear to be located at or near the solvent/protein interface and involved in absent or weak electrostatic interactions.

For I-$FABP_c$, the presence of a FA-protein electrostatic interaction by NMR was supported by our present interpretation of the 2.0 Å electron density map of crystalline *E. coli*-derived rat I-FABP [19]. The protein was crystallized with palmitate as its sole ligand. The bound ligand appears as a bent conformation probably reflecting the presence of several gauche bonds in its hydrocarbon chain. The principal bend in the hydrocarbon tail wraps around the indole side chain of Trp-82. But most importantly, the carboxylate moiety of the FA appears to be close to the guanidinium group of an arginine residue (Arg-106) and is located away from the protein/solvent interface [19]. Our current 2.0 Å model reveals that the carboxylate group of the bound FA is part of a five-member hydrogen bonding network consisting of 2 ordered water molecules, the δ-guanidinium moiety of Arg-106, and the side-chain oxygen of Gln-115 (see our accompanying article in this volume). The NMR chemical shift and ionization data for I-$FABP_c$, which were independently generated prior to the definition of its tertiary structure, provide additional support for the conclusion that the FA carboxylate group is located away from the solvent and involved in electrostatic charge interactions with the side chain of an arginine residue.

Unlike rat I-$FABP_c$, the three-dimensional structure of rat L-$FABP_c$ has not yet been determined. Rat L-$FABP_c$ contains an Thr residue at a position corresponding to Arg-106 of I-$FABP_c$, and this may, in part, explain the differences in ionization behavior observed for FA bound to rat I- and L-$FABP_c$. As noted above, the NMR results for L-FABP suggest that bound FA molecules exhibit a normal ionization behavior and are probably not involved in strong ion-pair interactions.

Based on the amino acid sequence homology, I- and L-FABP must share similar tertiary structures. The basic motif consists of two, five stranded antiparallel β-sheets arranged orthogonal to each other with the hydrophobic ligand located between the two sheets. This β-barrel topology is also shared by proteins binding other hydrophobic ligands including retinol, retinoic acid and bilin compounds. The apparent dilemma created by the present ^{13}C NMR results, in which L-FABP binds more than one FA whereas I-FABP binds only one, cannot be resolved without a detailed understanding of the tertiary structure of L-FABP. However, one possible explanation is that two FA are contained in a binding pocket analogous to that observed for I-FABP. This is not unreasonable since other structurally related hydrophobic ligand binding proteins bind compounds which are notably larger than fatty acids, i.e. retinoids and bile pigments. In those instances, the amino acid sequence may have evolved to leave more space in the binding pocket than is found in I-FABP.

Alternatively, L-$FABP_c$ may accommodate 1 FA in a binding site similar to that of I-$FABP_c$ while the sites and mechanisms of its interaction with the other FA are quite distinct. However, even the carboxyl moiety of the first molecule of FA bound to L-$FABP_c$ exhibited an NMR chemical shift and ionization behavior distinct from that for I-$FABP_c$. This observation suggests that even in the highest affinity site(s) on L-$FABP_c$, the FA carboxyl group is closer to the solvent than in the FA binding site on I-$FABP_c$.

The third major difference between I- and L-$FABP_c$ was in their pH sensitivity to FA dissociation, as monitored by changes in NMR peak intensities, and to conformational changes, as defined by changes in CD molar ellipticities. At and below physiological pH, L- (but not I-$FABP_c$) exhibited CD spectral changes concurrent with the

dissociation of its bound FA molecules. A similar pH-dependent dissociation of FA from L-FABP$_c$ was observed with preparations isolated from rat liver [18]. From the existing data, it is impossible to determine whether the CD changes observed for L-FABP$_c$ caused or resulted from the dissociation of FA.[1]

Since the FA binding properties of L-FABP$_c$ appear to be pH sensitive, even in the pH range normally found within cells, it is conceivable that the functional FA transport role of L-FABP *in vivo* may be regulated by small changes in intracellular pH. No such pH sensitivity is found with I-FABP$_c$. In addition, the observation that the carboxylate of the FA is closer to the solvent in L- vs I-FABP$_c$ may make it easier for L-FABP to accommodate amphiphilic ligands other than FA.

Functional implications for I- and L-FABP$_c$

Jejunal enterocytes contain abundant quantities of both I- and L-FABP$_c$, each representing approximately 2% of total cytosolic protein [20]. The small intestine absorbs and utilizes large quanitites of FA (approximately 300 mmol per day in humans on a typical Western diet; ref. [21]). However, unlike other tissues that utilize FA, e.g. heart, adipose tissue, and liver, the small intestine absorbs much larger quantities of monoacylglycerols (100 mmoles/day), lysophospholipids and bile salts (40 mmoles/day, each). Therefore, it is likely that enterocytes have developed a specialized mechanism for the cytoplasmic transport of these structurally diverse lipids.

By comparison, transport of these lipids in extracellular fluids essentially involves a single protein: serum albumin. Albumin's large size (67 kDa) and multidomain structure is well suited for simultaneous transport of a variety of structurally distinct amphiphiles including FA, bilirubin, bile acids, lysolecithin, drugs, etc. [22]. Its binding sites form two distinct structural and functional groups: 'FA-specific sites' that only bind long-chain non-esterified FA and 'non-specific sites' that bind a wide variety of amphiphiles including FA. In the *non*-specific sites, the carboxylate groups of bound FA are ionizable, solvent-accessible, and not involved in strong FA: protein electrostatic interactions [7]. In the specific sites, the FA do not exhibit a normal ionization behavior and probably interact strongly with cationic residues in the protein [7]. This division of binding sites into two functional types presumably serves a protective role, ensuring that the large quantity of non-esterified FA that enters the circulation each day is adequately solubilized and transported, while the smaller quantities of a number of other amphiphiles are also accommodated.

Based on the NMR results from this study and the above considerations, we hypothesize that a similar division of binding sites into two functional types occurs in the jejunal enterocyte cytosol, but in this case two separate proteins are involved: I- and L-FABP$_c$. Specifically, we have proposed [14] that I-FABP$_c$ exclusively binds and transports FA and is functionally analogous to the FA-specific sites on serum albumin. On the other hand, L-FABP$_c$ is proposed to bind and transport a variety of ligands such as FA, monoacylglycerols, lysophospholipids, and bile salts and is functionally analogous to the non-specific sites on serum albumin.[2] This hypothesis is illustrated and discussed in more detail elsewhere [14].

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[1] Since the tertiary structure for I-FABP$_c$ is very similar in the presence and absence of bound fatty acid [23], it appears that bound ligand is not required (at least for I-FABP$_c$) for stabilization of the β-clam structure.

[2] This statement is not meant to imply that the FA binding sites on I-, L-FABP$_c$, and serum albumin are structurally identical. FA binding sites on albumin are proposed to be formed by parallel rows of alpha-helices [21] whereas the binding site on I-FABP$_c$, as shown by x-ray crystallography, is formed by orthogonal beta-sheets [19].

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Assay of the binding of fatty acids by proteins: evaluation of the Lipidex 1000 procedure

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Key words: fatty acid-binding protein, fatty acid-binding assay, Lipidex 1000

Summary

Fatty acid (FA) binding by fatty acid-binding protein (FABP) is frequently monitored with the so-called Lipidex 1000 assay, in which protein associated and non-protein bound FA are separated by selectively binding the latter to Lipidex 1000. Careful evaluation of this assay showed that the use of aqueous FA solutions resulted in a marked decrease (60 to 70%) of FA concentration due to their aspecific binding to the surface of the test-tube used. In addition, solutions of rat heart FABP in the μmolar range also showed a concentration decrease up to 80% due to protein binding to the surface of the test-tube. Introduction of detergents, Triton X-100 or Tween 20, limited the FA loss to less than 20% and totally eliminated FABP adsorption. Kinetic parameters for the binding of [1-^{14}C]oleic acid by purified rat heart FABP, assayed in the presence of Triton X-100, were found to be similar to those assayed in the absence of detergent, when adequate corrections were made for losses of FA and FABP due to surface adsorption. Use of Tween 20 resulted in a substantial increase of the dissociation constant. The addition of 100 μM Triton X-100 to the assay medium considerably facilitates the determination of kinetic parameters of fatty acid-binding by proteins.

Introduction

In 1983, Glatz and Veerkamp [1] introduced a radiochemical procedure for the determination of the non-covalent binding of long-chain fatty acids (FA) by proteins. Lipidex 1000, a 10% substituted hydroxyalkoxypropyl derivate of Sephadex G-25, which shows appreciable affinity for hydrophobic substances in a temperature dependent manner, plays a crucial role in the assay procedure [2, 3]. At 37° C, Lipidex 1000 was found to remove both non-protein bound and protein associated FA from an aqueous solution, whereas at 0° C it only removed non-protein bound FA [1, 3]. This property made it possible to discriminate between free and protein bound FA in solutions containing fatty acid-binding proteins. The assay was initially developed to identify FABP during purification procedures and to study the FA binding capacities of various deal-buminized cytosol preparations [4]. At present, the assay is generally referred to as 'Lipidex 1000 assay' and is frequently used to monitor FA binding by purified fatty acid-binding proteins (FABP) in order to obtain apparent dissociation constants (Kd) and maximum binding (Bmax) values (e.g. [5–7]).

In the Lipidex 1000 assay, aqueous solutions of long-chain FA in the μmolar range are commonly used. Because of their amphiphilic character, FA have affinity for both hydrophobic and hydrophilic environments. As a result, FA in an aqueous environment will partially adsorb to the surface of the test-tube containing the FA solution as already mentioned previously [1]. Furthermore, at pH 7–9, FA will be present in two phases, a lamellar FA/

soap phase and the aqueous phase whereby the former will dominate [8, 9]. FA micelles will not be present at this pH interval [8]. Theoretically, the binding of FA to the surface of the reaction vial (e.g. glass or polypropylene) will result in a lower aqueous FA concentration, hence an underestimation of the bound/free (B/F) ratio and thus overestimation of the apparent dissociation constant (Kd). Besides FA, proteins are also known to adsorb to surfaces [10, 11] in a time and temperature dependent manner. Possible adsorption of FABP to the surface of the reaction vessel in the Lipidex assay will result in an underestimation of the value for maximum FA binding (Bmax).

The present study was carried out to determine the effects of adsorption of fatty acids and FABP to the surface of the reaction vessel on Kd and Bmax values as monitored by the Lipidex 1000 procedure. The effects of the utilization of detergents in the assay were also examined. Two commonly used non-ionic detergents, i.e. Triton X-100 and Tween 20, were included in the assay at various concentrations to minimize FA and FABP adsorption to the surface of the reaction vessel.

Materials and methods

Purified rat heart FABP was used in all experiments. Purification was carried out using gel filtration and ion-exchange chromatography essentially according to Paulussen *et al.* [5]. The overall yield of the protein was about 5%. Purity of the protein was confirmed with SDS polyacrylamide gel electrophoresis and isoelectric focussing using Phast System (Pharmacia LKB, Uppsala, Sweden). Amino acid analyses by HPLC [12] were carried out for precise FABP quantification. The coefficient of variation of amino acid analyses was in the order of 2% [5, 12]. Purified preparations were dialyzed against phosphate buffered saline (PBS), pH 7.4, overnight at 4° C and stored at − 20° C in polypropylene vials in a final concentration of 27.2 μM. When stored in this way, no detectable decrease of concentration or FA binding capacity of the samples was observed after 10 months.

[1-^{14}C]Oleic acid (specific activity 51.8 Ci/mol, 250 μCi in toluene) was purchased from Amersham International (Amersham, UK). The solution was evaporated under vacuum. Thereafter the oleic acid was redissolved in 10 ml absolute ethanol and stored in a final concentration of 0.48 mM at − 20° C. Lipidex 1000 was obtained from Packard Instrument Company Inc. (Downers Grove, Illinois, USA). Before use, Lipidex was exhaustively freed of methanol and stored as a 50% (v/v) suspension in 10 mM K-phosphate buffer, pH 7.4, at 4° C. Goat anti-rabbit IgG/horseradish peroxidase was obtained from Nordic (Tilburg, The Netherlands). All other chemicals were obtained from Merck (Darmstadt, FRG).

To measure the recovery of protein after Lipidex 1000 assay, FABP was quantified with an Enzyme Linked Immuno-Sorbent assay. For this, rabbits were immunized with purified rat heart FABP yielding antisera with a titer of 8000. FABP samples were directly coated onto PVC microtiterplates overnight at 4° C. After incubation with rabbit anti-rat heart FABP and subsequently with goat anti-rabbit IgG/horseradish peroxidase the FABP content was determined by measuring the oxidation rate of o-phenylene diamine in the presence of hydrogen peroxide. The detection limit of the assay amounted to 0.1 ng FABP.

Lipidex 1000 assay

The assay was performed in a 10 mM Tris-HCl buffer (pH 8.0) containing none or various concentrations of detergent as indicated. This buffer was also used to dilute FA and FABP stock solutions. [1-^{14}C]Oleic acid dilutions, ranging from 1 to 10 μM, were freshly prepared from the above mentioned ethanolic stock solution of 0.48 mM. Fixed amounts of FABP (136 ± 3 pmol) were used in each determination.

For the assay, 150 μl Tris-HCl buffer (pH 8.0), 50 μl FABP solution (2.72 μM) and 50 μl FA solution were mixed in this order in an 1.5 ml Eppendorf polypropylene reaction vial. After incubation for 15 minutes at 37° C, the vials were centrifuged for a few seconds to remove condense from the lid and subsequently placed on ice. From each vial

50 μl was taken and pipetted into a scintillation vial to assess the actual FA concentration in the aqueous solution. To the remaining volume, 50 μl of continuously stirred ice-cold Lipidex 1000 suspension was added and mixed 3–4 times on a vortex mixer during a 30 minutes incubation at 0° C. Finally, the vials were centrifuged (2 minutes, 10,000 g at 4° C) and 100 μl of supernatant was pipetted into scintillation vials to quantify the amount of bound FA. In some experiments an additional 10 μl was taken for FABP assessment in the solution using the above described ELISA. Radioactivity was measured with a Beckman LS 3801 scintillation counter (Beckman Instr. Inc., Fullerton, California, USA). Blank values were obtained for each FA dilution by measuring the radioactivity of incubations in which 50 μl Tris buffer without FABP was added. The measured blank values were subtracted from the FA binding data. Blank values increased when detergent concentration was elevated but never exceeded 3% of the total amount of radioactive FA added to each vial.

Data obtained were analyzed according to Zivin *et al.* [13] and visualized using Eadie-Hofstee plots in which the slope of the curve represents the apparent dissociation constant (Kd) and the Y-axis intercept the maximal FA-binding (Bmax). All values for FA binding are expressed as moles FA bound per mole of FABP.

Table 1. Recovery of [1-^{14}C]oleic acid in the aqueous medium at various detergent concentrations

Test-tube material	Detergent		[1-^{14}C]Oleic acid recovery (%)
Glass	None		25 ± 6
Polystyrene	None		65 ± 6
Polypropylene	None		42 ± 6
	Triton X-100	100 μM	84 ± 6*
		150 μM	82 ± 5*
		200 μM	84 ± 5*
	Tween 20	54 μM	76 ± 5*

Determinations were carried out in a total volume of 250 μl. Fatty acid concentration varied between 1 and 10 μM.
* Significantly different ($p < 0.05$) from values measured in the absence of detergent (Mean ± SD, n = 60).

Results

Data in Table 1 show that in glass, polypropylene and polystyrene vials a substantial amount of FA was lost from the aqueous solution when no detergent was present. In addition, FA loss appeared dependent on the material of which the vials are made (Table 1). The FA concentration decrease occurred instantaneously and independent of the temperature (data not shown). In polypropylene vials, addition of 100 μM Triton X-100 reduced the loss of FA to less than 20%. Elevation of the Triton X-100 concentration to 200 μM did not significantly further reduce the FA loss. It should be noted that all Triton X-100 concentrations were below its critical micellar concentration (0.24 mM). Utilization of Tween 20 gave comparable results.

Under our assay conditions FABP was also largely bound to the polypropylene vials. The recovery of FABP in the aqueous solutions amounted to 28 ± 4% (mean ± S.D. for 20 determinations) and was found to be dependent on the incubation time and temperature (data not shown). However, in the presence of detergent (100 μM Triton X-100), protein loss was completely eliminated during performance of the assay (data not shown).

Neglect of the binding of FA and FABP to the test-tube surface markedly influences the apparent dissociation constant (Kd) and maximal binding (Bmax). This is illustrated for a typical experiment in Fig. 1, in which empirically obtained data are

Table 2. Kinetic parameters for the binding of [1-^{14}C]oleic acid by rat heart FABP at various detergent concentrations

Detergent		Kd (μM)	Bmax
None		0.20 ± 0.15	1.20 ± 0.10
Triton X-100	100 μM	0.33 ± 0.02	1.11 ± 0.04
	150 μM	0.38 ± 0.03	1.03 ± 0.04
	200 μM	0.44 ± 0.02	1.13 ± 0.04
Tween 20	54 μM	0.79 ± 0.08	1.10 ± 0.07

Values and error limits of Kd and Bmax were calculated from 20 (no detergent) or 60 (all other cases) independent fatty acid-binding measurements in polypropylene vials made for each condition (see Fig. 2 for further details). Parameters were calculated using the corrected FA and FABP concentrations.

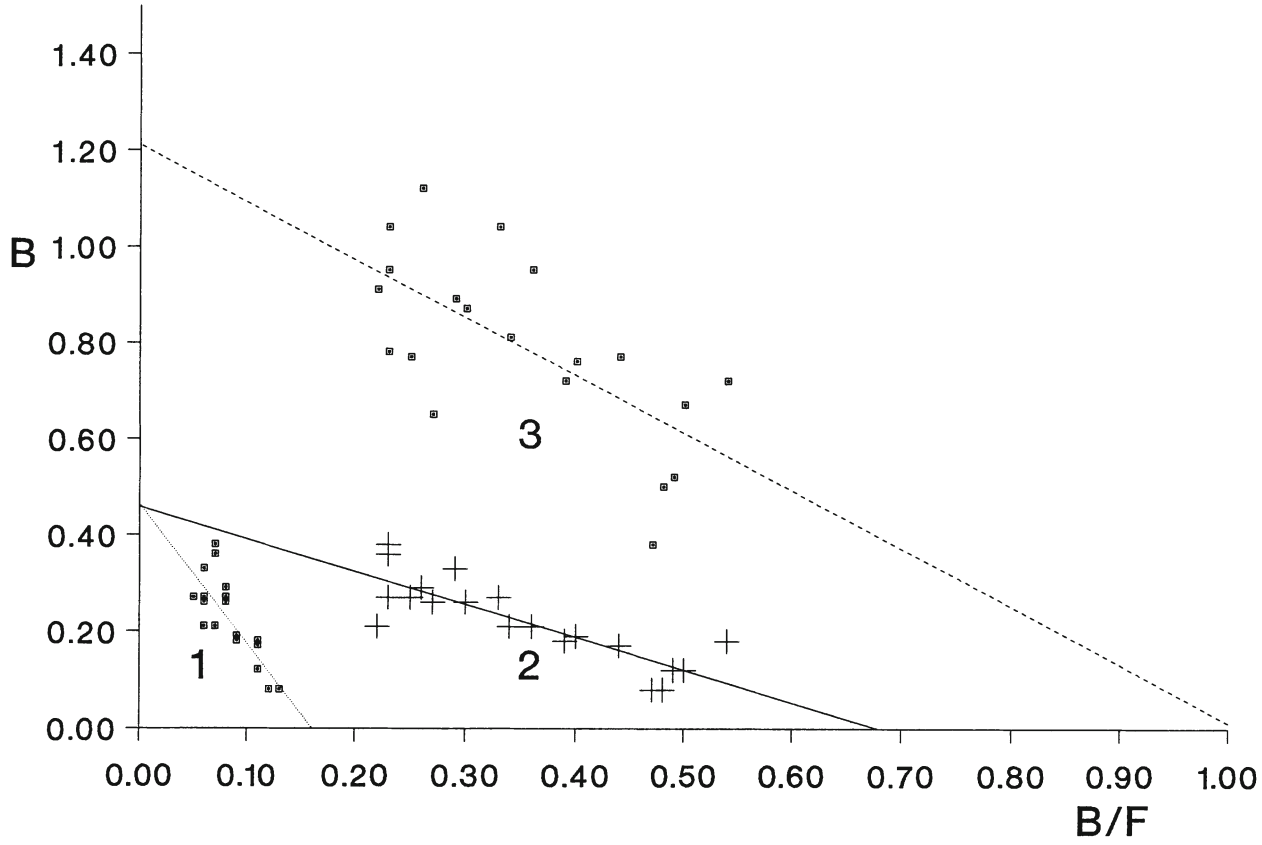

Fig. 1. Eadie-Hofstee plot of oleic acid-binding by rat heart FABP in the absence of detergent: Effect of the binding of FA and FABP to the polypropylene test-tube. Actual concentrations of FA were measured radiochemically, those of FABP by ELISA. The results of a representative experiment are shown. In graph 1 both FA and FABP losses were assumed to be negligible (Kd = 1.60 ± 0.30, Bmax = 0.46 ± 0.05, r = 0.79). In graph 2 a correction was made for the loss of FA only (Kd = 0.37 ± 0.05, Bmax = 0.46 ± 0.04, r = 0.84). Graph 3 shows the plot with the actual aqueous concentrations of FA and FABP after the correction of losses due to adsorption to the surface of the test-tube (Kd = 0.20 ± 0.15), Bmax = 1.20 ± 0.10, r = 0.69).

presented using the Eadie-Hofstee plot technique [13]. The advantage of this representation technique is that both Kd and Bmax values and their error limits are estimated in a direct manner. When FA and FABP losses are not considered (graph 1), the Kd is substantially overestimated and the Bmax underestimated. A correction made for FA loss alone (graph 2) still leads to erroneous values. Therefore, it is important to eliminate both FA and FABP adsorption to the surface of the test-tube during the Lipidex 1000 assay as much as possible.

The influence of the presence of Triton X-100 or Tween 20 on the kinetic parameters of oleic acid-binding by FABP, calculated on the basis of the actual FA and FABP concentrations is illustrated in Fig. 2 (data given in Table 2). The detergents did influence the apparent Kd to some extent but did not significantly affect Bmax values (Table 2). The Kd value slightly increased when the detergent concentration was elevated. The affinity of FABP for FA apparently decreases when a detergent is present in the assay. The largest increase in Kd value was observed when Tween 20 was used in the assay at a concentration of 54 μM.

Discussion

The present findings indicate that for the assay of the binding of fatty acids by FABP in aqueous

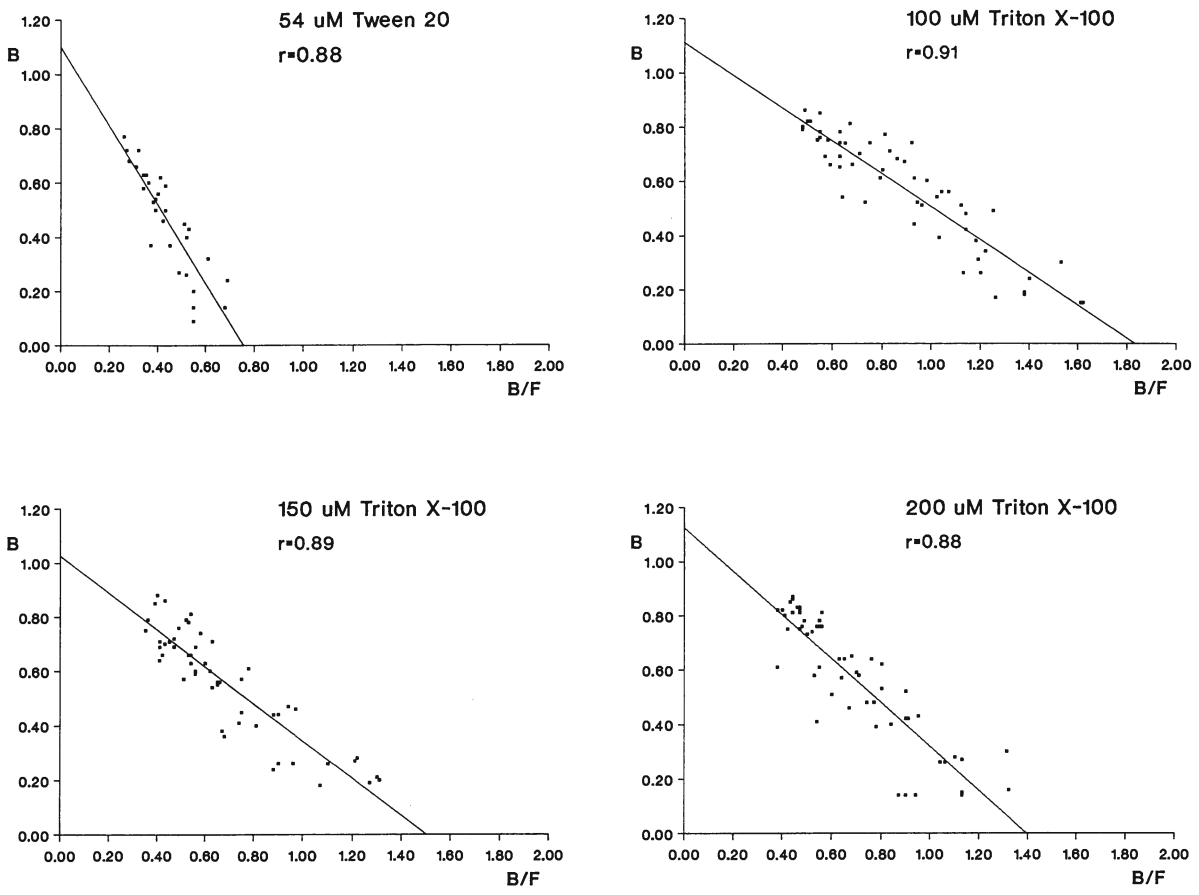

Fig. 2. Effect of detergents on the binding of oleic acid by rat heart FABP. Eadie-Hofstee plots are given for determinations in the presence of 100–200 μM Triton X-100 and 54 μM Tween 20, all carried out in polypropylene vials as described in Materials and Methods. The results of representative experiments are shown. Kinetic parameters calculated from the graphs displayed are given in Table 2.

media, adsorption of both FA and FABP to the surface of the test-tube is remarkably high and, therefore, seriously hampers the correct calculation of Kd and Bmax values. The effects of Triton X-100, commonly used in membrane protein purification, and Tween 20, applied in many enzyme linked immunosorbent assays, were investigated. These detergents significantly decreased the loss of FA from the aqueous solution and completely eliminated the loss of FABP. Kinetic parameters calculated from binding studies in the presence of Triton X-100 appeared similar to those calculated from studies in which no detergent was used but in which corrections were made for FA and FABP adsorption to the test-tube. Thus, addition of detergent to the medium considerably facilitates the assay of the binding of fatty acids by FABP.

The problem of binding of FA to the surface of the test-tube was already noted in the first description of the Lipidex 1000 assay [1], but the surface adsorption of protein (FABP) was not yet discussed before. Using polyethylene vials and 10-minute incubations at pH 7.4, in this previous study the recovery of [1-^{14}C]palmitate from the assay mixture was found to be 90–95% [1], significantly higher than our present observations. Hence, in the absence of detergent, the actual aqueous FA concentration depends to a large extent on the experimental conditions and, possibly, on the type of fatty acid employed.

The presence of a detergent in the Lipidex 1000

assay did influence the apparent Kd value (Table 2), especially in case of Tween 20 at a relative low concentration. A feasible explanation for this phenomenon can be offered when the molecular structure of Tween 20 is taken into account. According to Sacchettini *et al.* [14], the free carboxylate group of long-chain fatty acids plays a role in the binding of FA by FABP. Because Tween 20 contains a C12 fatty acid chain with a free carboxylate group, it may bind to FABP in a similar way as FA do. In analogy to enzyme kinetics, this phenomenon may be called competitive inhibition resulting in increased Kd values. The Bmax value, however, will not change because the inhibitory effect of Tween 20 can be eliminated by elevating the concentration of FA.

The explanation given above is not applicable to the small increase in apparent Kd value observed in the presence of Triton X-100, as this detergent lacks a fatty acid group. Possibly, at high concentrations Triton X-100 binds to hydrophobic regions on the FABP molecule resulting in a small conformational change of the protein molecule, thereby causing a decrease of the affinity of FABP for FA.

Despite the fact that Bmax values obtained in this study in the absence and presence of detergent all were slightly but significantly higher than 1 ($p < 0.05$), except for the data obtained with 150 μM Triton X-100, the linear relationships observed (Fig. 2) support the notion that only one single FA binding site is present on rat heart FABP (see ref. [15] for a review). In an assay with oleic acid-loaded phospholipid liposomes the Bmax for oleic acid has been reported to be 0.7 [5] and 2 [16]. However, in these studies an additional surface for FA binding viz. phospholipid bilayers [17] was present which may have affected the maximal FA binding by FABP.

The results of the our study indicate the difficulty of interpretation of previously reported values of Kd and Bmax as obtained with the Lipidex 1000 assay and may partially explain the large variation of kinetic parameters reported till now [15]. To our knowledge, adequate corrections for surface adsorption of ligands and proteins have not been made in any of these studies. Generally, because the concentration of non-protein bound FA was always presumed not to decrease (at least the reverse was not indicated by the authors) the B/F ratio will have been underestimated resulting in an overestimation of the Kd values calculated.

In conclusion, application of the Lipidex 1000 assay to obtain kinetic parameters of FA binding by proteins requires the measurement after incubation of the actual concentrations of FA and protein. The use of labelled FA ensures an easy and accurate determination of their total aqueous concentration, but protein measurements are less accurate and rather laborious as their low concentrations require immunochemical techniques. Thus, for a convenient and yet significant performance of the assay, the addition of detergent to the assay medium is indicated as it will prevent FABP adsorption and limit FA adsorption to the surface of the test-tube. It is recommanded to include Triton X-100 at a final concentration of 100 μM in the assay. Apparent Kd values obtained in the presence of Triton X-100 will probably be slightly higher than the true values, but binding stoichiometry can be accurately assessed.

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Fatty acid-binding protein from human heart localized in native and denaturing two-dimensional gels

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Key words: fatty acid-binding protein, human heart, native 2-D gel

Summary

A group of low molecular weight fatty acid-binding cytosolic proteins, $FABP_c$, with high abundance in heart, liver, skeletal muscle, intestine and adipose tissue, are anticipated to play a role in long-chain fatty acid metabolism in these tissues. Recently, a $FABP_c$ with M_r 15 kDa has been purified from human heart muscle and found to be present in levels 2–4% of cytosolic proteins of human heart myocytes. In the present study two-dimensional gel electrophoresis under native and denaturing conditions has been used to characterize $FABP_c$ from human heart and this protein is found to be a major protein of human heart myocytes. The pI of $FABP_c$ from human heart was found to be about 5.3 under native conditions and about 6.5 in the presence of 9 M urea.

Introduction

Fatty acid-binding proteins (FABPs) have been purified from various human tissues: heart [1, 2], placenta [3], and adipose tissue [4]. The cardiac type fatty acid-binding protein, $H\text{-}FABP_c$, constitutes 2–4% of cytosolic proteins in human heart myocytes [1] and polyclonal antibodies prepared against this protein react with an abundant fatty acid-binding protein from human skeletal muscle [5]. In the rat $H\text{-}FABP_c$ shows similar high abundance in heart cytosol 5–6% [6] and mRNA for $H\text{-}FABP_c$ is found at levels of 63% of heart levels in slow-twitch muscle [7]. Human $H\text{-}FABP_c$ has been sequenced and found to contain 132 amino acids with a M_r of 14768 [8]. A physiological role for $H\text{-}FABP_c$ seems to be as storage and transport protein for long chain fatty acids in the cytosol [9], transporting these poorly soluble molecules [10] from plasma membrane locations to intracellular sites for fatty acid metabolism. Such a role makes sense in heart muscle where β-oxidation of long chain fatty acids accounts for 80% of energy production [11].

There is some evidence for heterogeneity of human heart $FABP_c$ as sequence data [8] and amino acid analysis [1] of this protein do not show identical amino acid composition and, additionally, two different pI values of 7 [2] and 5.3 [1] have been reported.

The aim of the present study is to characterize $FABP_c$ in human heart by two-dimensional gel electrophoresis according to O'Farrell [12]. This technique was applied in two versions, first a denaturing method as described by Bravo and Celis [13] capable of resolving 250–300 protein spots in the high speed supernatant from human heart, and secondly a non-denaturing method according to Manabe *et al.* [14] allowing fatty acids to remain bound to fatty acid-binding proteins during the entire electrophoresis. $H\text{-}FABP_c$ was found to be present as the second most abundant soluble pro-

tein in human heart myocytes, following myoglobin, and the pI of this protein was observed to increase in the presence of 9 M urea.

Materials and methods

Purification of human heart FABP

Human heart muscle was obtained 14–44 hours post mortem from the Institute of Forensic Medicine at Aarhus University. No major difference in yield of H-$FABP_c$ was observed between samples obtained at different times after death. Adipose and fibrous tissue was removed and muscle tissue frozen in liquid nitrogen and stored at − 80° C. Preparation of a 100,000 g fraction from heart muscle tissue and purification of FABP from this high speed supernatant was done essentially as described by Unterberg *et al.* [1].

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis under denaturing conditions was made according to O'Farrell [12]. The equipment and detailed protocol used in the present work have been described by Celis *et al.* [13, 15]. In short, sample was solubilized in lysis buffer [12] containing 9.8 M urea, 100 mM DTT and applied to a first dimension isoelectric focusing gel. This gel was made in 2 mm i.d. borosilicate glass tube and gel composition 4% T, 5% C, 1.6% servalyte 5–7 (Serva, FRG), 0.4% ampholyte 3.5–10 (LKB, Sweden), 9.8 M urea. Following focusing for 18 h at 400 V, tube gels were equilibrated in 1% SDS, 100 mM DTT and mounted on top of a second dimension SDS-PAGE gel with stacking gel composition 5% T, 5% C, 0.1% SDS, 120 mM Tris-HCl pH 6.8 and running gel composition 18% T, 0.5% C, 0.1% SDS, 375 mM Tris-HCl pH 8.8. Two-dimensional gel electrophoresis under native conditions was made according to Manabe *et al.* [14], using the same equipment as for denaturing gels. Samples were solubilized in 40% sucrose and applied to a first dimension isoelectric focusing gel with similar composition as used for denaturing 2-D electrophoresis, except for urea which was omitted in native 2-D gels. Focusing at 400 V for 18 h was done at + 4° C as higher temperature caused bubbles to form in the gel. The isoelectric focusing gel was mounted, without equilibration, onto a second dimension PAGE gel with stacking gel composition 5% T, 5% C, 120 mM Tris-HCl, pH 8.8 and running gel composition 10% T, 0.5% C, 375 mM Tris-HCl pH 8.8 and electrophoresed overnight at room temperature.

Staining procedures

Denaturing 2-D gels were silver stained using an ammoniacal silver stain protocol [16]. Although sensitive, this method failed to give visible staining of albumin and FABP in native gels, on the contrary these proteins tend to resist staining by this method (data not shown). Therefore Coomassie Brillant Blue R (Sigma) was used for staining native 2-D gels.

Autoradiography

Addition of radioactive fatty acids to protein samples containing fatty acid binding proteins, prior to protein analysis by isoelectric focusing or PAGE, as described by Unterberg *et al.* [1] was employed using native 2-D gel electrophoresis for protein separation. For one experiment 2 μCi of ^{3}H-palmitate in 2 μl 99% ethanol was added to 200 μg of high speed supernatant or 100 μg of purified FABP in 100 μl water. Next 50 mg sucrose was added to a final concentration of 40% and samples applied to a first dimension isoelectric focusing gel. Following electrophoresis in the second dimension gels were dried directly without staining using Bio-Rad gel drier. Gels for autoradiography were mounted with Kodak X-ray film and exposed at − 80° C for 2–8 days.

Fatty acid content

Determination of non-esterified fatty acids (NE-

Fig. 1. Denaturing two-dimensional gel electrophoresis of 100,000 g supernatant of homogenized human heart muscle. Abundant cytosolic proteins myoglobin (2) and H-FABP$_c$ (3) are marked with arrows. The positions of major plasma proteins from extracellular fluid albumin (1), α_1-antitrypsin (arrows), haptoglobin β chain (arrow heads) are indicated for reference. Approx. 200 μg of protein was applied to the gel, which has been silverstained according to Tunón and Johansson [16].

FA) bound to our purified H-FABP$_c$ preparation was done using an enzymatic method from Wako (Wako Chemicals GmbH, Neuss, FRG). One measurement could be done using 300 μg or 50 μl of a 400 μM FABP solution. Usually, 0.6–0.9 mol FA per mol H-FABP$_c$ was found.

Results

Denaturing two-dimensional gel electrophoresis of the 100,000 g supernatant of homogenized human heart tissue, Fig. 1, shows about 200–300 spots, being primarily cytosolic proteins. However, plasma proteins abundant in extracellular fluid are not removed from the tissue by brief washing in buffer prior to homogenization and therefore such proteins are also present in the gel picture. The pI ranges from about 4 to 7.5 and M_r from 10 kDa to 70 kDa, only few protein spots are visible above this molecular weight (data not shown). By comparison with a 2-D gel of purified human heart FABP$_c$ (Fig. 3a), the spot(s) corresponding to this protein can be identified and marked at pI 6.5 (major spot) and pI 6.2 (minor spot). Myoglobin is found at M_r 17 kDa, pI 7.5. It is seen that in fact the major spot of FABP$_c$ is very prominent among cytosolic proteins and appears to be the second most abundant protein in human heart cytosol, following myoglobin. Major plasma proteins, albumin, α_1-antitrypsin, and haptoglobin β-chain are identified by comparison with 2-D gels of human serum and are marked with arrows in Fig. 1. Non-denaturing or native two-dimensional gel electrophoresis of the 100,000 g supernatant of homogenized human heart tissue, Fig. 2, shows about 50 spots, corresponding primarily to cytosolic proteins with a few contaminating proteins from interstitial fluid. Albumin from blood plasma and extracellular fluid, identified by comigration with purified human serum albumin, is a major spot in this gel and the fastest migrating protein, found at

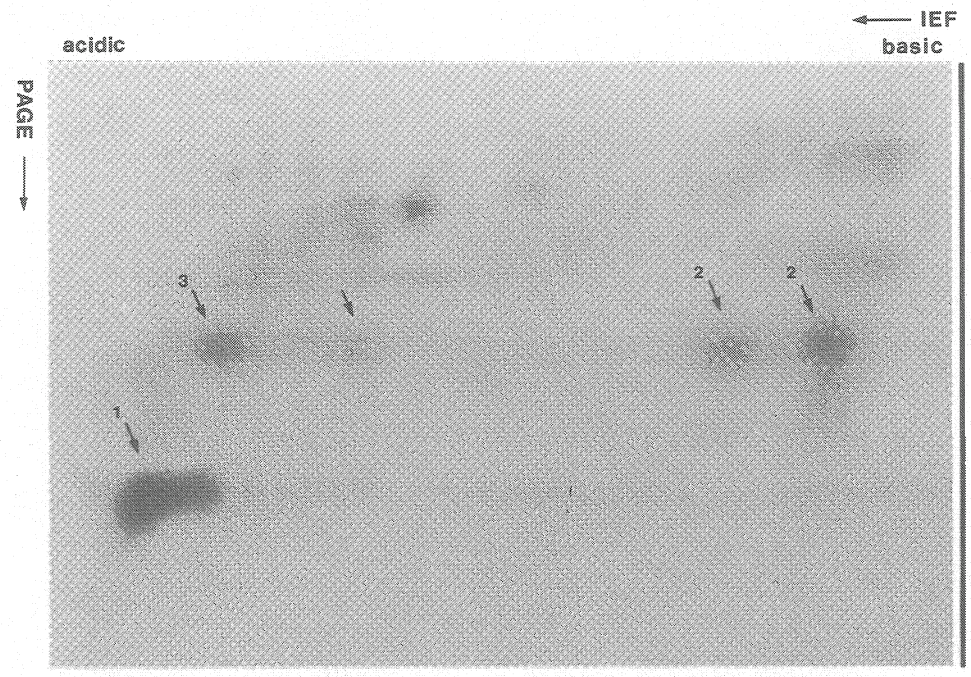

Fig. 2. Native two-dimensional gel electrophoresis of 100,000 g supernatant of homogenized human heart muscle. Abundant cytosolic proteins myoglobin (2) and H-FABP$_c$ (3) are marked with arrows. The position of plasma albumin (1) from extracellular fluid is marked for reference. Approx. 300 μg of freeze dried high speed supernatant was applied to the gel, which has been stained with Coomassie Blue.

pI around 4.7. Myoglobin, identified by its red color visible during the entire electrophoretic procedure is found as two spots around pI 7. The more acidic of these two spots is likely to be the native protein, myoglobin, and the more basic protein, metmyoglobin.

By comparison with a similar 2-D native gel of purified FABP$_c$ from human heart, Fig. 3b, FABP$_c$ is identified as the major spot migrating above the albumin spot at pI around 5.3. A minor spot with similar migration in the second dimension, but focusing at higher pI, is found in both gels. FABP$_c$ appears to be a very abundant cytosolic protein in this type of gel and comparable in staining intensity with myoglobin. Denaturing and native two-dimensional gel electrophoresis of purified FABP$_c$ shows our preparation from human heart to contain two protein spots. A major spot and a minor spot constituting less than 5% hereof. At present the relationship between these two spots is not known. However, in denaturing gels, Fig. 3a, the major spot focuses at pI around 6.5 in accordance with reports by Glatz *et al.* [2] and the minor spot focuses at a slightly lower pI, whereas in native gels, Fig. 3b, the major spot focuses at pI around 5.3 in accordance with reports by Unterberg *et al.* [1] and the minor spot focuses at slightly higher pI under these conditions. A similar tendency to show an increase in pI upon isoelectric focusing in the presence of urea is also known for plasma albumin and observed in the present work (compare Fig. 1 and Fig. 2).

Native 2-D gel electrophoresis of purified human heart FABP$_c$ which have been loaded with ^{3}H-palmitate prior to electrophoresis, Fig. 3c, revealed comigration of radioactive fatty acids and FABP$_c$. No radioactivity was found to comigrate with the minor spot of our FABP$_c$ preparation.

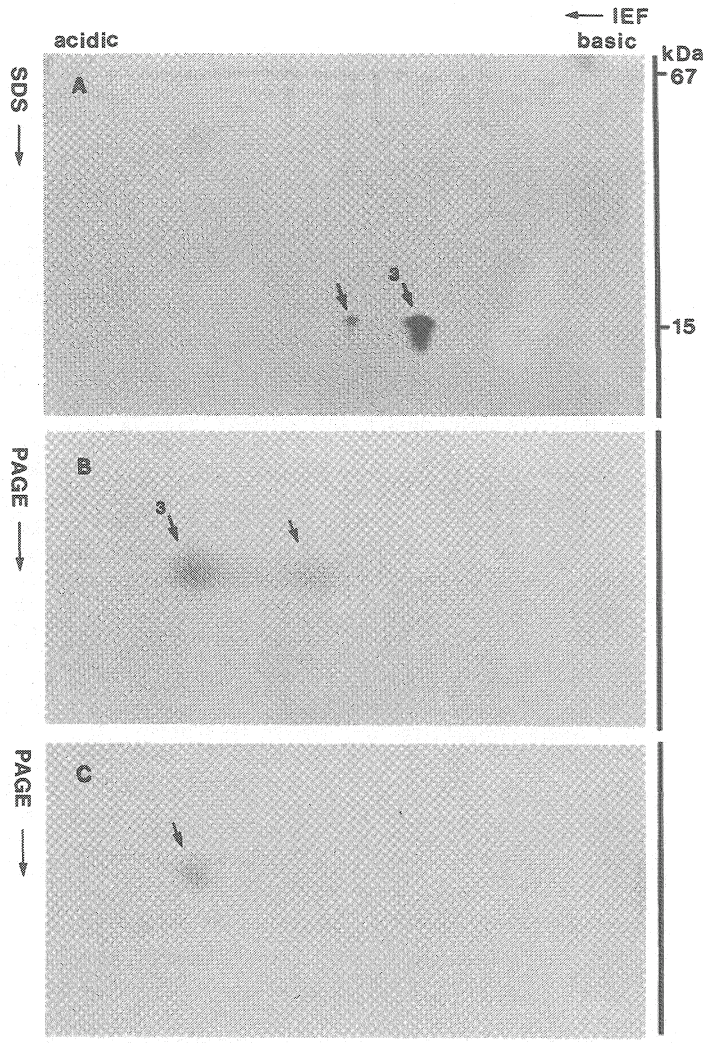

Fig. 3a. Denaturing two-dimensional gel electrophoresis of purified $FABP_c$ from human heart muscle. Approx. 20 μg of H-$FABP_c$ was applied to the gel which has been silverstained according to [16]. The pI of the major spot is about 6.5.
Fig. 3b. Native two-dimensional gel electrophoresis of purified $FABP_c$ from human heart muscle. Approx. 40 μg of H-$FABP_c$ was applied to the gel which has been stained by Coomassie Blue. The pI of the major spot is about 5.3.
Fig. 3c. Autoradiography of native 2-D gel of purified $FABP_c$ from human heart. 100 μg of H-$FABP_c$ was mixed with 2 μCi of ^{3}H-palmitate prior to isoelectric focusing. Second dimension PAGE gel was dried without staining and exposed for 2 days at − 80° C. The arrow points to the position of H-$FABP_c$ in 2-D native gel from a parallel run.

Discussion

β-Oxidation of long chain fatty acids is the major source for energy production in the heart [6] and also in skeletal muscle rich in slow-twitch fibers. However, the solubility of the most abundant plasma long chain free fatty acids, palmitate and oleate, has been estimated to be less than 0.4 nM in phosphate buffer at pH 7.4 [10]. This concentration is by far too low to sustain the metabolic rate of free fatty acid oxidation in the heart and phrase a requirement for the existence of fatty acid-binding proteins in the cytosol to facilitate transport and enhance solubility of these molecules. In human

heart H-FABP$_c$ is a potential candidate for a protein with this function as it is very abundant and binds 0.8–1.0 mol fatty acid per mol FABP during the entire purification protocol, even in the presence of excess albumin (present work). Transport of long chain fatty acids from extracellular location, primarily binding areas on interstitial albumin, to intracellular locations are anticipated to occur by free diffusion across the plasma membrane [17] and facilitated entrance by binding to a plasmalemmal FABP, H-FABP$_{PM}$ [18]. Such a fatty acid transport chain bears resemblance to oxygen transport by the hemoglobin/myoglobin couple and in the human heart myoglobin and FABP$_c$ levels are similar intracellularly (present study) as are albumin and hemoglobin levels extracellularly. Along this line of view recent reports by Pette *et al.* ([19, 20] and references therein) showing increased levels of both extracellular albumin and intracellular FABP$_c$ upon electrostimulation of fast-twitch muscle add evidence in support of a concerted action of FABP$_c$ and albumin in transporting fatty acids from extracellular to intracellular locations. Currently, the cause of heterogeneity of our FABP$_c$ preparation is not known or whether it is caused by an impurity or an isoelectric charge variant. Interestingly, Jagschies *et al.* [21] found two isoelectric isoforms of H-FABP$_c$ from bovine heart at pI 4.9 and 5.1 in ratio 3 : 1, respectively.

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Revision of the amino acid sequence of human heart fatty acid-binding protein

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Key words: fatty acid-binding protein, mass spectrometry, sequence, human heart

Summary

Cardiac-type fatty acid-binding protein (cFABP) from human heart muscle of three individuals was isolated and characterized as pI 5.3-cFABP. The proteins were structurally analyzed by tryptic peptide mapping, application of plasma desorption time-of-flight mass spectrometry and amino acid sequencing. All three preparations of human heart FABP, having 132 amino acids, differed from the published sequence [Offner *et al.* Biochem J 251: 191–198, 1988] in position 104, where Leu is found instead of Lys, and in position 124, where Cys is found instead of Ser.

Introduction

The three types of fatty acid-binding proteins (FABPs) that have been distinguished with respect to their source of first isolation are intestinal, hepatic and cardiac FABP. This classification is justified by their immunological behavior as fatty acid-binding proteins from different species crossreact only within one type [1].

Cardiac-type FABP (cFABP) from human heart was first isolated and characterized by Unterberg *et al.* [2] and shown to be a 15 kDa protein with an isoelectrical point of 5.3 (pI 5.3-cFABP). Recently, Offner *et al.* [3] published the primary structure of this protein, which exhibited high homology to rat (89.4% identity, [4]) and bovine cFABP (87.9% identity, [5]). However, in contrast to the amino acid composition given by Unterberg *et al.* [2], Offner *et al.* [3] did not report cysteine in this protein. As we had previously established peptide mapping for both cFABP isoforms of bovine heart cytosol (pI 4.9- and pI 5.1-cFABP) [6] and had available an expeditious and reliable method for identification of peptides, namely plasma desorption time-of-flight mass spectrometry (PDMS) [7], we reconsidered the amino acid sequence of human heart FABP.

Experimental procedures

Isolation and characterization of cFABPs. Human and bovine heart FABP were isolated as described in references [2] and [6], respectively, and characterized by SDS-PAGE (20%) with subsequent Western blotting as well as anion exchange HPLC on Mono Q (see below).

Modification of cFABP with vinylpyridine. pI 5.3-cFABP (20 nmol) was reduced with 2 μmol dithiothreitol (DTT) in 6 M guanidinium.HCl/20 mM Tris.HCl/1 mM EDTA, pH 7.5, and incubated with

2 μl vinylpyridine (25° C, 10 min) in a final volume of 200 μl. Oxygen was excluded by flushing all media with nitrogen. Upon fivefold dilution with water a precipitate was formed which was then washed with 0.1 M NH_4HCO_3 and digested with trypsin as described below.

Enzymic cleavage. Prior to digestion native or modified cFABP was taken up in water, subjected to heat denaturation and simultaneously suspended by sonication, and was then digested with 2% (w/w) trypsin in 0.1 M NH_4HCO_3, pH 8.0 (37° C, 2 h).

HPLC separations. Chromatography was performed on a Kontron HPLC-system connected with a Mono Q HR 5/5 (Pharmacia) column for proteins and with the following reverse phase columns for peptides: Nucleosil RP 18 (10 μm, 300 Å, 8 × 125 mm), Nucleosil RP 4 (10 μm, 300 Å, 4.6 × 125 mm, Macherey & Nagel) and Dynospheres PD 102-RE (4.6 × 30 mm, Dyno Particles), the latter especially suited for hydrophobic molecules.

Plasma desorption time-of-flight mass spectrometry (PDMS). Tryptic peptides from HPLC-runs were lyophilized and dissolved in 0.1% TFA/20% methanol. Aliquots (~ 200 pmol) were applied onto a nitrocellulose coated aluminized mylar foil by the spin technique [8] and bombarded with fission fragments from the ^{252}Cf source of a BIO-ION 10 K mass spectrometer. The acceleration voltage was set at 16 kV and 500 000 primary ions were recorded. Reduction of disulfides was carried out *in situ* with 3 nmol DTT at 37° C for 2 h in a humid chamber. All masses are given as average molecular mass.

Sequence analysis and peptide nomenclature. Tryptic peptides were subjected to automated Edman degradation in a Knauer 810 sequencer. They were sequentially numbered according to primary structure (T-1 to T-14) or according to elution order in reverse phase HPLC of respective FABP-samples (F1–1 to F1–12 for FABP-1 and F2-0 to F2–11 for FABP-2).

Results

Characterization of pI 5.3 cFABP

Both human and bovine heart FABP had a similar molecular weight in SDS-PAGE and, after subsequent Western blotting, reacted with the polyclonal antibody against bovine cFABP [1]. They also behaved similar on the Mono Q ion exchange column, where bovine pI 4.9- and human pI 5.3-cFABP exhibited identical retention times. In contrast, the isoforms of bovine cFABP, pI 4.9- and pI 5.1-cFABP, could be separated on this anion exchanger [6].

Peptide mapping

Tryptic peptides of human and bovine cFABP were separated on a reverse phase C_{18} column. The elution pattern of pI 5.3-cFABP peptides (Fig. 1A) also indicated strong similarities to that of bovine pI 4.9-cFABP (data not shown), as expected from the high homology. Due to the characteristic absorbance at 280 nm (arrows in Fig. 1) the tryptophan and tyrosine containing peptides could be easily assigned to the known peptide map of bovine pI 4.9-cFABP, as published elsewhere [6]. In particular, Tyr^{128} and Tyr^{19} containing peaks F1–1 and F1–9 exhibited the same retention time as their bovine counterparts. Yet the Trp^{97} containing peptide F1–10 eluted slightly and the Trp^{8} containing N-terminal peptide F1–12 considerably later than the corresponding peptides from bovine cFABP. The latter was expected from the exchange of Val^{5} in pI 4.9-cFABP against Leu, resulting in a more hydrophobic peptide.

Characterization of peptides

In order to identify all tryptic peptides of pI 5.3-cFABP unequivocally and to align them with the reported sequence [3], we employed plasma desorption time-of-flight mass spectrometry [7]. This method enabled us to determine the molecular mass of peptides with an accuracy of 0.1%, thus giving sufficient information to prove the overall amino acid composition of a certain peptide. The mass of the singly charged, protonated molecular ion of the N-terminal peptide (MH^+ = 1077 Da) is

in accordance with the mass calculated from the sequence [3] and confirms the existence of an acetyl blocking group. Table 1 summarizes the masses of all peptides. To our surprise we found two deviations from expected masses, namely for peptides T-12 and T-14.

The first deviation can be seen from the mass spectrum shown in Fig. 2A. For peptide T-12 we found a molecular mass of 1203 Da that could not be fitted into the sequence reported by Offner *et al.* [3]. But from the fragmentation peak MH^+-130, which was due to the loss of the indol side chain of tryptophan, and from the molecular mass, which indicated a decapeptide, we concluded that this peptide represented positions 97 to 106. Upon automated Edman degradation the sequence was determined to

W D G Q E T T *L* V R

in full support of the mass found by PDMS. The mass of the corresponding peptide, as calculated from the published sequence [3], is 964 Da and no peptide with this mass was found (Table 1).

The second deviation occurred in the peptide presumed to cover positions 113 to 126. Due to its very hydrophobic nature it could only be recovered by reverse phase HPLC on the Dynospheres column, where it eluted with the same retention time as peptide T-13 from bovine pI 4.9-cFABP (data not shown). The mass spectrum showed a molecular mass of 1496 Da and not 1482 Da as expected from the published sequence [3]. Furthermore a peak of nearly the same height with the mass 2995 Da was detected and tentatively interpreted as a dimer formed via disulfide bridge. This interpretation was supported by the observation that *in situ* reduction with DTT indeed caused the disappearance of the 2995 Da molecular ion (Fig. 2B). The molecular mass of monomeric T-14, its retention time in reverse phase HPLC, identical to the bovine counterpart, and the presence of a reducible dimer can only be explained by the presence of Cys^{124} instead of Ser^{124} in human cFABP. Hence human cFABP peptide T-14 and bovine cFABP peptide T-13 are identical. Our results are summarized in Fig. 3, where the revised sequence and the alignment of the tryptic peptides are presented.

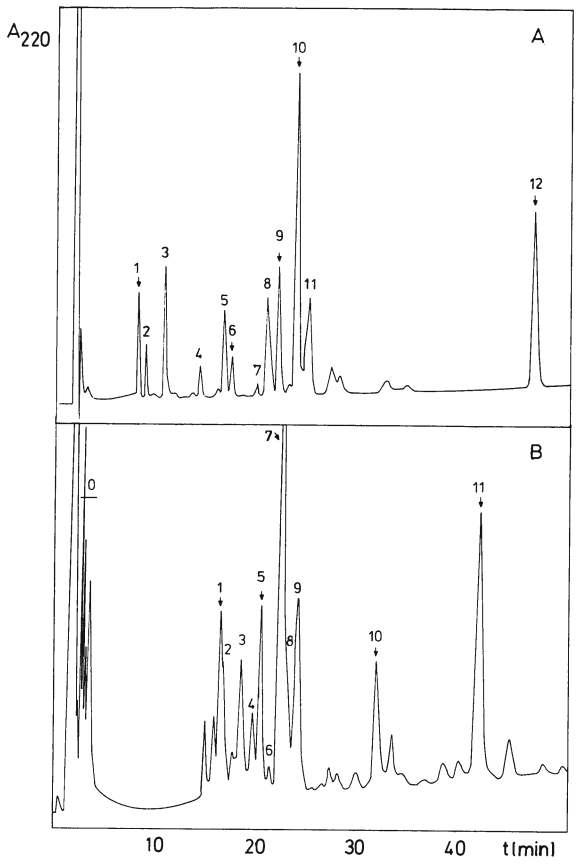

Fig. 1. Separation of tryptic peptides from pI 5.3-cFABP by reverse phase HPLC. A gradient of 50% isopropanol in 0.1% trifluoroacetic acid was applied (3 min 0%, 45 min to 45%, 12 min to 60%, 1.5 ml/min). Detection at 220 nm (0.2 AUFS), arrows indicate peptides with strong absorbancy at 280 nm (0.01 AUFS). A, peptides from 150 μg FABP-1 on RP 18 column; B, peptides from 150 μg vinylpyridinylated FABP-2 on RP 4 column.

At this stage the question arose, whether the differences observed between reported and revised sequences were due to individual polymorphism or not. To solve this problem, we examined human heart FABP from two other individuals (FABP-2, FABP-3). The masses of the corresponding tryptic peptides are also given in Table 1 and fitted into the sequence as specified in Fig. 3 for FABP-2. They clearly support the results obtained for FABP-1 and suggest that human heart FABP has, inde-

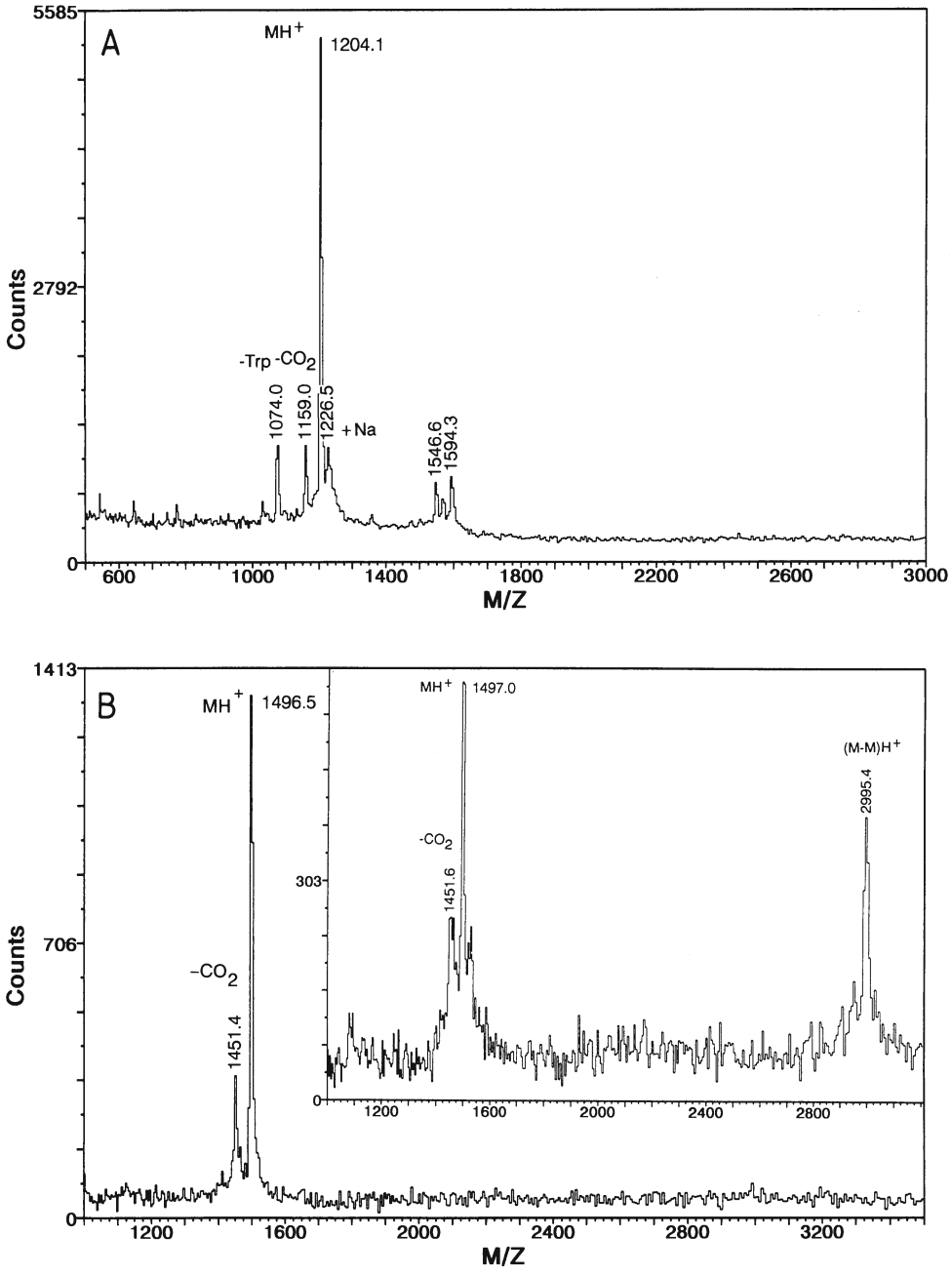

Fig. 2. Mass spectra of tryptic peptides from pI 5.3-cFABP. A, peptide T-12 from FABP-1; B, peptide T-14 from FABP-1, reduced *in situ* with 3 nmol DTT, the mass spectrum of the unreduced peptide T-14 is inserted.

pendently of the individual source, the amino acid sequence presented here.

Further proof for Cys^{124} in T-14 was obtained by modification of the protein (FABP-2) with 4-vinyl-pyridine. In the corresponding tryptic peptide map on a reverse phase C_4 column (Fig. 1B) a new peak (F2–10) with a characteristic absorbancy at 260 nm and a mass of 1604 Da eluted, as expected for vinyl-

pyridinylated T-14. The sequence of this peptide, was determined to

L I L T L T H G T A V C T R

corroborating the foregoing data.

Discussion

In the literature some controversy exists on the amino acid sequence of rat heart FABP. Hitherto three different sequences from five laboratories are available, even in a revised sequence [9] two discrepancies to the cDNA [4, 10] can be found. The cDNA derived sequence is supported by a recent report [11], in which rat cFABP peptides are examined by amino acid analysis.

Here we demonstrate that the primary structure of human heart FABP from three different individuals differs in two positions from the published structure [3]. Namely in position 104 Leu is found instead of Lys. The misinterpretation may be caused by cleavage after Leu^{104} due to chymotryptic activity of trypsin. This seems plausible, because FABPs are quite resistant to enzymic degradation, and large quantities of trypsin as well as long incubation times are often applied. Furthermore in position 124 we observed Cys instead of Ser. Since cysteine is difficult to detect in sequencing and amino acid analysis without prior alkylation, we modified pI 5.3-cFABP with 4-vinylpyridine. This modification was not applied by Offner *et al.* [3]. The presence of cysteine and its potential for disulfide bridge formation is supported by data of Nielsen *et al.* [12], who observed dimers of human cFABP in denaturing two-dimensional gel electrophoresis. These dimers could be monomerized upon reduction with DTT.

The revised sequence results in a higher homology of human cFABP to bovine pI 4.9-cFABP (89.4% identity). This particular bovine cFABP isoform also contains Asp in position 98 [6], whereas in the other isoform (pI 5.1 cFABP) of bovine heart cytosol Asn^{98} is found as the only difference. But, though both bovine isoforms can be separated upon anion exchange chromatography on Mono Q, bovine pI 4.9-cFABP and human pI 5.3-cFABP

Table 1. Summary of mass spectrometric data

Peptide	Residues in sequence	M_r calc.	M_r by PDMS		
			FABP-1	FABP-2	FABP-3
T-1	1–9	1077[1]	1076	1077	nd
T-2	10–14	561	560	561	nd
T-3	15–21	932	932	932	nd
T-4	22–30	907	906	nf	nd
T-5	31–44	1546	1546	1564[2]	nd
T-6	45–52	873	nf	873	nd
T-7	53–58	720	719	719	nd
T-8	59–65	838	838	838	nd
T-9	66–78	1467	1466	1467	nd
T-10	82–90	889	888	889	nd
T-11	91–96	737	736	736	nd
T-12	97–106	1204	1203	1204	1204
T-13	107–112	673	673	673	nd
T-14	113–126	1498	1496	1604[3]	1498
T-15	127–130	539	539	540	nd

[1] N-acetylated peptide.

[2] The higher M_r is due to oxidation of Met ($MH^+ + 16$).

[3] M_r of the vinylpyridinylated T-14, (M_r vinylpyridine = 105.1).

nd, not determined; nf, not found.

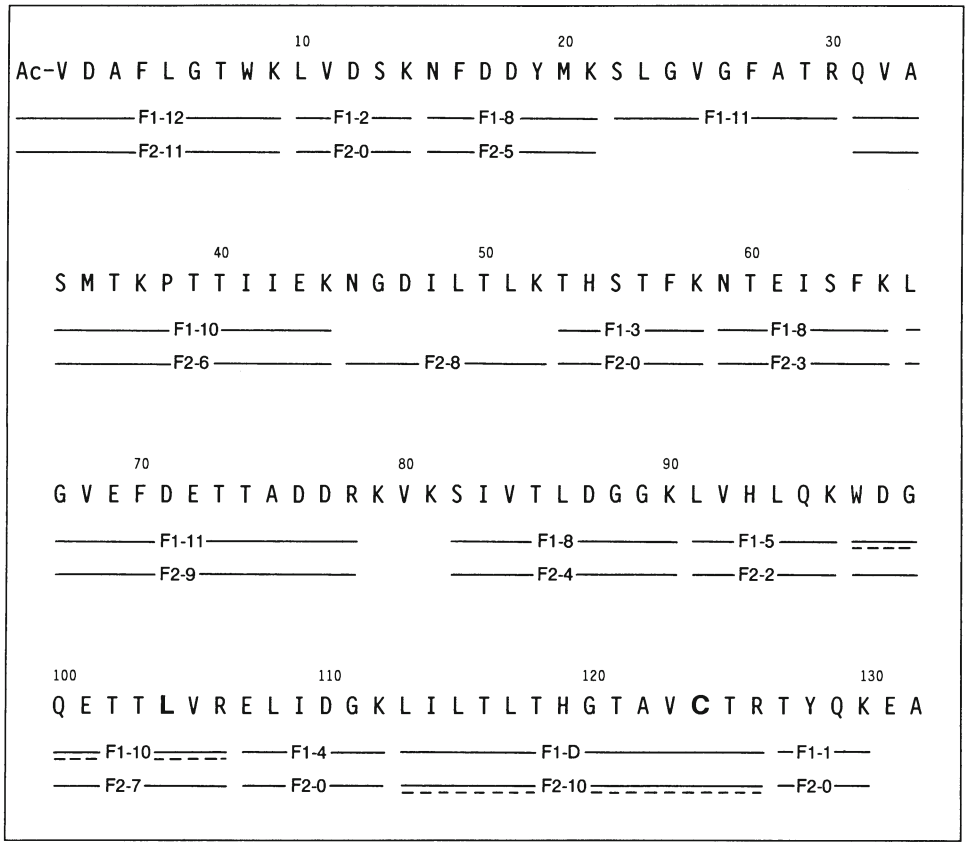

Fig. 3. Revised sequence of pI 5.3-cFABP. Differences to published sequence [3] are shown in bold type. Aligned peptides from FABP-1 and FABP-2 were characterized by PDMS (——) and sequencing (– – –) and denoted according to their elution order as shown in Fig. 1A. Peptide T-14 was recovered from the Dynospheres column (F1-D). The four peptides from FABP-2 not bound by the RP 4 column (Fig. 1B, peak 0) were rechromatographed on the RP 18 column prior to mass determination and are all denoted F2-0.

exhibit identical retention times. Hence the elution order seems to be dominated by the negative charge of the surface-located Asp^{98} of those proteins.

In contrast to bovine cFABP, no isoforms were detected for human cFABP. In isoelectric focusing only one FABP was found by Offner *et al.* (pI 5.3) [3] as well as by Paulussen *et al.* (pI 5.2) [13], in agreement with Unterberg *et al.* [2]. Based on this it is unlikely that the sequences presented here and in ref. [3] correspond to different isoforms.

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The chemical modification of cysteine-69 of rat liver fatty acid-binding protein (FABP): a fluorescence approach to FABP structure and function

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Key words: Hepatic-FABP, fluorescence, chemical modification

Summary

Hepatic-FABP was labelled at cysteine-69 with the fluorescent environmentally sensitive reporter group AEDANS. The labelled protein had an emission maximum at 465 nm indicating that cysteine-69 was buried in a non-polar environment. The modified protein was still able to bind ligands such as oleic acid, oleoyl CoA and haem. The affinity of AEDANS-FABP for haem was unaltered as compared with the native protein indicating that cysteine-69 must be remote from the ligand binding site. The binding of oleic acid did not significantly perturb the fluorescence emission spectrum of the fluorescent reporter group suggesting that there are not large conformational changes in the region of cysteine-69 on fatty acid binding. The binding of stoichiometric amounts of oleoyl CoA was accompanied by a small fluorescence enhancement which suggests that fatty acyl CoAs may interact with other regions of the FABP molecule not involved in fatty acid binding.

Abbreviations: FABP – Fatty Acid-Binding Protein, AEDANS – 5-[2-{(Acetyl)amino}Ethyl]Aminonaphthalene-1-Sulphonic Acid, IAEDANS – 5-[2-{(Iodoacetyl)Amino}Ethyl]aminonaphthalene-1-Sulphonic Acid, DTNB – 5,5′Dithiobis-(2-Nitrobenzoic acid)

Introduction

Rat liver FABP contains a unique cysteine at position 69, a feature that is not conserved in other members of the FABP family. Because free cysteine residues will selectively react with a wide range of sulphydryl reagents we have adopted an approach to FABP structure and function which involves the chemical modification of this residue. Initial studies involved the reaction of cysteine-69 with DTNB and this work established that the cysteine is free but buried within the protein [1]. Moreover, DTNB-modified FABP was still able to bind fatty acids suggesting that cysteine-69 was remote from the ligand binding site of the protein [1]. Thus cysteine-69 is an ideal site of attachment of for an environmentally sensitive reporter group that might respond to changes in protein conformation on ligand binding and provide information about the structure and function of this protein.

In this paper we describe the modification of cysteine-69 with the fluorescent polarity-sensitive probe, 1,5-AEDANS which as its iodoacetyl derivative (1,5-IAEDANS) can be used to selectively modify sulphydryl residues. The fluorescent aminonaphthalene sulphonate group is particularly sensitive to polarity changes and as such is well suited to study a soluble protein that binds non-polar ligands.

The results confirm that cysteine-69 is buried within a non-polar environment and is not involved in ligand binding. Moreover ligand binding does

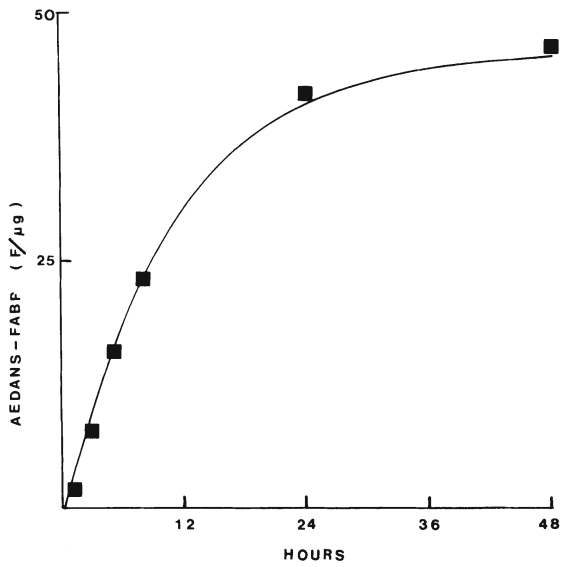

Fig. 1. Time course of the incorporation of AEDANS into FABP. FABP was incubated with 1,5-IAEDANS at room temperature in the dark. Aliquots were removed at the times shown and purified by gel permeation h.p.l.c. The protein concentration and the fluorescence of the purified sample were determined and results were expressed as fluorescence/µg.

not cause significant perturbation of the reporter group indicating that if there are conformational changes on ligand binding, the changes do not have a major effect within the region of cysteine-69.

Experimental procedures

Materials

1,5-IAEDANS was obtained from the Aldrich Chemical Company, 1,5-AEDANS-mercaptoethanol was prepared by the method of Hudson and Weber [2]. Oleic acid, oleoyl CoA and oestrone sulphate were obtained from the Sigma Chemical Company.

Preparation of FABP

Hepatic FABP from rat liver was prepared by methods described previously [1].

Labelling of FABP with 1,5-IAEDANS

FABP (0.25 mg/ml) in 0.1 M potassium phosphate buffer pH 7.2 was reacted with 1,5-IAEDANS (0.23 mg/ml) in the dark at room temperature. At the end of the incubation the labelled protein was purified using G-25 Sephadex or by gel-permeation h.p.l.c. using a TSK G2000SW column (7.5 mm × 600 mm).

Fluorescence measurements

All fluorescence measurements were performed using a Perkin Elmer LS3B fluorimeter and disposable plastic cuvettes. In experiments involving haem the fluorescence intensity was corrected for inner filter effects.

Results

The reaction of cysteine-69 with 1,5-IAEDANS

The time course of labelling of cysteine-69 of liver FABP is shown in Fig. 1. The reaction was slow and incubation for 24 hours was required to incorporate 1 mol of 1,5-AEDANS per mol of protein. This slow rate of reaction is consistent with cysteine-69 being buried within the protein. The stoichiometry of incorporation after 24 hours was confirmed by spectral analysis of the modified protein following purification. The absorbance at 280 and 340 nm were compared and the concentration of protein and probe were quantified using molar extinction coefficients at 280 and 340 m of 9800 and 7000 $l \cdot mol^{-1} \cdot cm^{-1}$ respectively. It was calculated that 1.01 mols of AEDANS had been incorporated per mol of FABP.

That the AEDANS group was indeed located at cysteine-69 was confirmed by assessing the DTNB reactivity of the modified protein. After 24 hours incubation with 1,5-IAEDANS the DTNB reactivity of the modified protein was only 7% that of the native protein indicating that 93% of cysteine-69 had been modified. In addition, quenching of the fluorescence of AEDANS-FABP by acrylamide results in a linear Stern-Vollmer plot with an intercept of 1.0. This results shows that the labelling reaction has produced a single class of fluophores with all probe residues equally accessible to collisional quenching [3] consistent with a

unique site on the protein being modified. Therefore it may be concluded that FABP had been labelled with 1 mol of AEDANS and over 90% of the label was located at cysteine-69.

Spectral properties of AEDANS-FABP

The fluorescence emission spectrum of purified AEDANS-FABP is shown in Fig. 2. The protein was highly fluorescent with an emission maximum at 465 nm. This wavelength is indicative of the probe being located in a non-polar environment and appears to be the lowest wavelength of emission so far reported for a protein labelled with this probe. When the spectral properties of the protein bound probe are compared with those of mercaptoethanol modified with 1,5-AEDANS measured in increasing concentrations of ethanol the emission wavelength is consistent with an environment corresponding to 80% ethanol (Table 1). These results indicate that the probe is buried within the non-polar core of the protein and consistent with the low DTNB reactivity of the protein already described [1].

Table 1. The effect of ethanol on the fluorescence properties of AEDANS-Mercaptoethanol. The emission spectrum of AEDANS-mercaptoethanol (6×10^{-8} M) was recorded in aqueous solutions containing increasing proportions (v/v) of ethanol. Excitation was at 337 nm.

Solvent	Fluorescence emission maximum (nm)	Relative intensity
Water	495	100
20% Ethanol	488	187
40% Ethanol	481	259
60% Ethanol	475	347
80% Ethanol	465	426
100% Ethanol	453	435

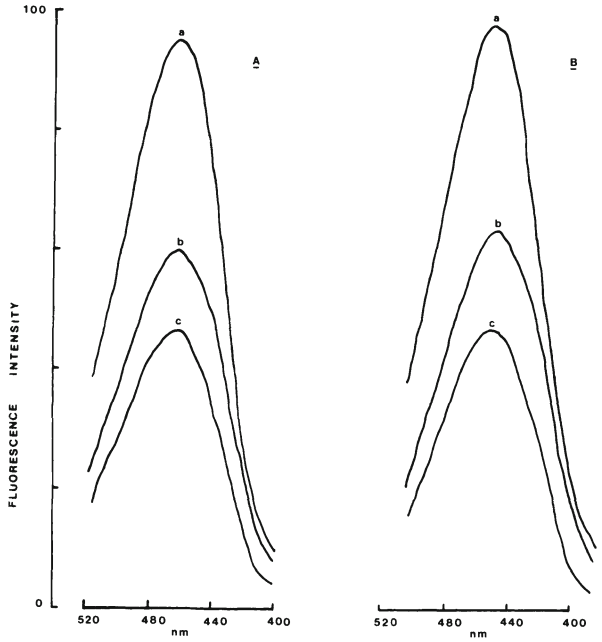

Fig. 2. Fluorescence emission spectra of AEDANS-FABP. The fluorescence emission spectra of (a) AEDANS-FABP (2.15×10^{-7} M); (b) AEDANS-FABP plus haem plus oleic acid or oleoyl CoA (5×10^{-7} M); (c) AEDANS-FABP plus haem (2×10^{-7} M); were recorded in 0.1 M potassium phosphate buffer pH 7.2. Excitation was at 350 nm. 2A, spectra for oleic acid expt.; 2B, spectra for oleoyl CoA expt.

The effect of ligands on the fluorescence properties of AEDANS-FABP

The capacity of the AEDANS modified FABP to bind ligands was confirmed using the haem, a ligand with a known high affinity for FABP [4–7]. Because of spectral overlap, if haem binds it will quench the fluorescence of AEDANS-FABP and this is shown in Fig. 2. Analysis of the quenching curve (Fig. 3) gave an apparent Kd for haem binding to hepatic FABP of 2×10^{-7} M which compares very well to values of 2.5×10^{-7} M [4] and 1.5×10^{-4} M [5] previously reported for haem binding to the unmodified protein.

This result confirms that the modified protein is still able to bind with high affinity and is consistent with the observation that the DTNB-modified protein is still able to bind ligands [1]. No change in the wavelength of emission maximum was observed on

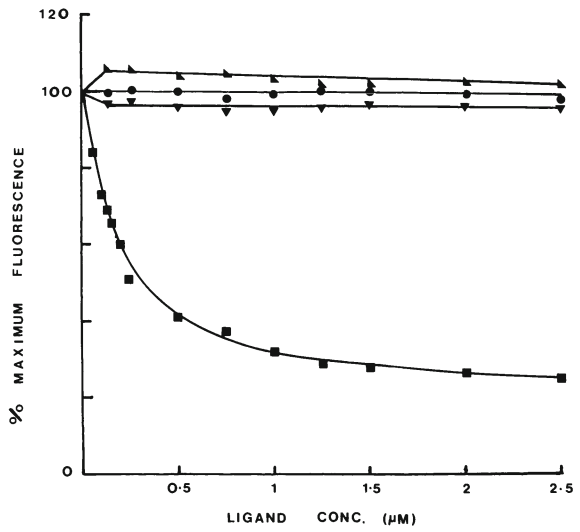

Fig. 3. Effect of various ligands on the fluorescence intensity of AEDANS-FABP. The ligand was titrated into a solution of AEDANS-FABP (2.15×10^{-7} M) and the change in fluorescence was recorded. In each case the ligand was added as a 10^{-4} M solution and curves are corrected for any effect of solvent. Oleic acid and oestrone sulphate were dissolved in methanol. ■——■, haem; ▼——▼, oleic acid; ◀——◀, oleoyl CoA; ●——●, oestrone sulphate.

adding heam indicating that the haem was not causing any major perturbation of the protein in the region of cysteine-69 on binding to FABP.

The effect of fatty acids (oleic acid) and fatty acyl CoA on the spectral properties of AEDANS-FABP was also determined (Fig. 3). Oleic acid had no effect on either the intensity of emission or the wavelength of maximum emission (data not shown) suggesting that fatty acids do not produce a large conformational change in FABP on binding, or at least, not a change that can be detected in the region of cysteine-69. That the fatty acid was binding to the modified protein was confirmed by the observation that oleic acid was able, by competition, to reduce haem binding to the protein and thus able to reverse the fluorescence quenching observed with haem (Fig. 2A).

With the addition of oleoyl CoA (Fig. 3) a small but significant fluorescence enhancement (~5%) was observed after the addition of stoichiometric amounts of this ligand. Again oleoyl CoA was able to reduce the quenching produced by haem (Fig. 2B) indicating that the oleoyl CoA was competing with haem for the ligand binding site on FABP.

Discussion

The precise physiological role of FABP's remains elusive despite the discovery of a number of different but related proteins in different tissues that bind fatty acids [8]. The fact that different tissues have evolved different proteins strongly suggests that these proteins may have a more specific function than simply binding fatty acids within the cell. A complete understanding of the function of FABPs requires a detailed knowledge of protein structure and, in particular, possible changes in protein structure on binding to ligands and/or subcellular components.

In this paper we describe a fluorescence approach to studying the structure and function of FABP and to this end we have made use of the fact that hepatic-FABP contains a unique cysteine at position 69 that is amenable to chemical modification. In a previous communication we have demonstrated that this cysteine was remote from the fatty acid binding site and appeared to be buried within the protein such that it was not readily accessible to the sulphydryl reagent DTNB [1]. We have now labelled the cysteine uniquely with the environmentally sensitive reporter group AEDANS and this probe has provided a variety of information on the structure and function of this protein.

The emission maximum of the probe shows a considerable blue shift in wavelength compared to the probe in buffer consistent with cysteine-69 being buried in a non-polar environment within the protein. There is now strong evidence to suggest that FABPs and related proteins may have a similar 'clam shell-like' structure made up to 10 anti-parallel β-strands organised into two orthogonal β-sheets [9–11]. Therefore direct comparison with the crystal structure of intestinal-FABP which is now available is possible [10, 11]. It is very encouraging to note that the equivalent position in native intestinal-FABP is phenylalanine-69 and this residue is directed into the core of the protein. More-

over this phenylalanine is in proximity to other non-polar residues namely phenylalanine-63, tyrosine-71, tryptophan-83 and phenylalanine-94. The corresponding positions in liver-FABP are predicted to be phenylalanine-63, leucine-71, valine-83 and phenylalanine-95 [8] consistent with cysteine-69 also being buried in a non-polar environment. This data provides evidence to support the concept that although the homology between FABP from rat liver and intestine is low, their crystal structures are very similar.

We have used the high affinity ligand, haem, to demonstrate by fluorescence quenching that not only is this ligand still able to bind to AEDANS-modified FABP but also, using competitive displacement of the haem, that oleic acid and oleoyl CoA are still able to bind to the modified FABP. The apparent remoteness of cysteine-69 from the fatty acid binding site is surprising and would not have been predicted by comparison with the crystal structure of intestinal-FABP which contains bound palmitic acid. However, in intestinal-FABP arginine-106 has now been identified as the site of interaction with the fatty acid carboxylate group and there is *not* an equivalent arginine in hepatic-FABP. Alternatively arginine-122 in hepatic FABP is conserved both in intestinal-FABP and the P2 myelin protein while the crystal structure of the P2 protein [9] suggests that this arginine is involved in carboxylate binding. Therefore in hepatic-FABP as with the P2 protein from myelin the fatty acid may bind in a different location within the hydrophobic core of the protein more remote from position 69 than that demonstrated for intestinal-FABP.

We have been unable to detect any conformational change on binding of either oleic acid or oestrone sulphate to the protein as judged by a change in fluorescence signal from the reporter group. This result has now been shown to be consistent with the observation that for intestinal-FABP the crystal structures of the apo-protein and the protein containing bound fatty acid are essentially identical indicating that there is an absence of conformational change on fatty acid binding [12].

Of particular interest is the fact that the addition of a stoichiometric amount of fatty acyl CoA (oleoyl CoA) to AEDANS-FABP results in a small but significant fluorescent enhancement. This result suggests that the more bulky oleoyl CoA cause a conformational change in the protein possibly because it binds to FABP in a different manner to oleic acid involving other regions of the protein as well as the fatty acid binding site.

Although we have been unable to detect conformational change in the protein on fatty acid binding it is possible that conformational changes do occur on binding of FABP to other cellular proteins or membranes. Such interactions would be expected if hepatic-FABP had a specific targetting role or regulatory role within the cell and these potential interactions will be the subject of further investigations with AEDANS-FABP.

Acknowledgements

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A comparison of heart and liver fatty acid-binding proteins: interactions with fatty acids and possible functional differences studied with fluorescent fatty acid analogues

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Key words: fatty acid, fatty acid-binding protein, lipid transport, binding protein, fluorescence

Summary

Fatty acid-binding proteins (FABP) are distinct but related gene products which are found in many mammalian cell types. They are generally present in high abundance, and are found in those tissues where free fatty acid (ffa) flux is high. The function(s) of FABP is unknown. Also not known is whether all FABP function similarly in their respective cell types, or whether different FABP have unique functions. The purpose of these studies was to assess whether different members of the FABP family exhibit different structural and functional properties. Two fluorescent analogues of ffa were used to compare the liver (L-FABP) and heart (H-FABP) binding proteins. The propionic acid derivative of diphenylhexatriene (PADPH) was used to examine the physical properties of the ffa binding site on L- and H-FABP, as well as the relative distribution of ffa between FABP and membranes. An anthroyloxy-derivative of palmitic acid, 2AP, was used to monitor the transfer kinetics of ffa from liver or heart FABP to acceptor membranes, using a resonance energy transfer assay. The results demonstrate that the ffa binding sites of both FABP are hydrophobic in nature, although the L-FABP site is more nonpolar than the H-FABP site. Equilibration of PADPH between L-FABP and phosphatidylcholine (PC) bilayers resulted in a molar partition preference of $>20:1$, L-FABP : PC. Similar studies with H-FABP resulted in a PADPH partition preference of only 3 : 1, H-FABP : PC. Finally, the transfer of 2AP from H-FABP to acceptor membranes was found to be 50-fold faster than transfer from L-FABP. These studies demonstrate that important structural and functional differences exist between different members of the FABP family, and therefore imply that the roles of different FABP may be unique.

Abbreviations: FABP – Fatty Acid-Binding Protein; L-FABP – Liver FABP; H-FABP – Heart FABP; SUV – Small Unilamellar Vesicle; PADPH – 3-[p-(6-Phenyl)-1,3,5-Hexatrienyl]-phenylpropionic acid; 2AP – 2-(9-Anthroyloxy)Palmitic acid; Q – Quantum yield; τ_F – Fluorescence lifetime

Introduction

Many mammalian tissues process large quantities of unesterified fatty acids (ffa). A family of low molecular weight proteins which bind ffa, the fatty acid binding proteins (FABP), has been purified from these tissues. FABP are relatively abundant at both the protein and mRNA levels. The developmental expression of several FABP has been shown to be different, as are the patterns of FABP tissue distribution. Interestingly, some cell types contain more than one form of FABP (for reviews see 1–4).

At present, the precise function(s) of the FABP

remains unknown. Although their unique distributions and patterns of expression might suggest that different FABP have different functions, it is not yet known whether such differences exist, or whether all the FABP play similar roles in their respective tissue types.

The liver and heart FABP are among the least similar members of the FABP family. These FABP share only 36% sequence homology, as compared to the 62% identity found between heart and adipocyte FABP [5]. Moreover, L- and H-FABP have almost no cross-tissue distribution. Whereas L-FABP is found in liver and intestine, H-FABP is most abundant in heart and skeletal muscle [4]. Finally, it is of interest that ffa in the heart are almost exclusively utilized for oxidation, while liver ffa may be used for both anabolic and catabolic processes.

In this report, two fluorescent analogues of ffa were used to compare the structural and functional properties of L-FABP and H-FABP. The propionic acid derivative of diphenylhexatriene (PADPH) was used to examine the physical properties of the ffa binding sites, and the relative distribution of ffa between each of these FABP and membranes [6]. An anthroyloxy-derivative of palmitic acid, 2AP, was used to monitor the kinetics of ffa transfer from liver or heart FABP to acceptor membranes using a resonance energy transfer assay [7]. The results demonstrate that the ffa binding site of L-FABP is more hydrophobic than the H-FABP binding site. In addition, PADPH was found to bind with a molar partition preference of $>20:1$, L-FABP: phosphatidylcholine (PC), compared with only 3: 1 for H-FABP: PC. Finally, the transfer of 2AP from H-FABP to acceptor membranes was observed to be markedly faster than transfer from L-FABP.

Experimental procedures

FABP purification

L-FABP, purified and delipidated as described previously [8, 9], and H-FABP, purified by the method of Said and Schultz [10], were generously provided by Dr. Nathan M. Bass. Protein concentrations were determined colorimetrically [11] and corrected by factors of 0.6 and 0.8 for L- and H-FABP, as previously discussed [8].

Vesicle preparation

Small unilamellar vesicles (SUV) were prepared from 90 mole% egg phosphatidylcholine (EPC) and 10 mole% N-(7-nitro-2,1,3,-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE), or 25 mole% bovine brain phosphatidylserine (PS), 65 mole% EPC and 10 mole% NBD-PE. Sonication, ultracentrifugation and storage of PC- and PS- containing SUV was at 4° C and 15° C, respectively [7]. Lipids were purchased from Avanti Polar Lipids (Birmingham, AL).

Fluorophores and fluorescence measurements

The fluorophores used in these studies were 3-[p-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (PADPH) and 2-(9-anthroyloxy)palmitic acid (2AP), both from Molecular Probes (Eugene, OR). Excitation wavelengths were 360 and 383 nm and emission wavelengths 430 and 450 nm for PADPH and 2AP, respectively.

Emission spectra and time-dependent measurements were made using an SLM 8000C fluorescence spectrophotometer. Data were collected in photon counting mode. Excited state lifetimes (τ_F) were obtained using an SLM 4800C subnanosecond fluorescence spectrophotometer. Measurements were made at 18 mHz as previously described [12], using a Corning 3-74 400 nm long pass emission filter. Fluorescence polarization was determined with the SLM 8000C, using the 3-74 filter to monitor emission in the perpendicular direction (SLM-Aminco, Urbana, IL).

Fluorescence quantum yields (Q) were determined using quinine sulfate in 0.1 N H_2SO_4 as reference fluorophore with $Q = 0.7$. Excitation was at 352 nm. Radiative and nonradiative decay rates (k_f and k_{nr}) were calculated using the experimental values of Q and τ_F, as previously described [12].

FABP (5 to 14 μM) were incubated with 1 to 2 μM PADPH at ambient temperature. PADPH was added from concentrated stock solution in dimethylformamide (DMF), with final DMF concentration $<1\%$ (v/v). For determinations of Q, a

ratio of ≥ 10 : 1 FABP : PADPH was used, and it was assumed that all ligand was bound. Buffer was 0.1 M sodium phosphate, 50 mM NaCl, pH 7.4. Intensities of FABP alone, SUV alone, or fluorophores in buffer at the concentrations used in these experiments, were negligible.

Fatty acid transfer assay

A fluoresscence resonance energy transfer assay [7] was used to directly monitor the transfer of 2AP from FABP to membranes. FABP were incubated with 2AP for 10 to 15 min at ambient temperature. Equal volumes of FABP-2AP were then mixed with NBD-PE-containing vesicles using a Hi-Tech SFA-11 stopped flow apparatus (Hi-Tech Scientific, UK), and 2AP transfer from FABP to membranes was monitored by the decrease in 2AP fluorescence with time, at 23° C. 2AP (donor) added directly to the NBD-PE-containing vesicles is > 98% quenched by energy transfer to the fluorophore (acceptor), so that during the observed time course the fluorescent signal derives only from FABP-bound 2AP. The final transfer mixture contained 5 to 7 μM FABP, 0.5 to 1.5 μM 2AP and 125 to 150 μM phospholipid.

The decrease in 2AP fluorescence over time was analyzed by fitting the data to an exponential function, as previously described [7]. Fit quality was assessed by the normalized χ^2 with standard deviations determined by the square root of the number of photons. The transfer data obtained were best fit by a single exponential function. (A second component, which accounted for < 10% of the total 2AP quenched, was observed in some experiments with H-FABP. This small, very slow decrease in intensity was determined to be due not to 2AP transfer but to fluorophore bleaching. The use of neutral density filters in the excitation path largely prevented this problem).

PADPH distribution between FABP and membranes

To determine the relative partition of PADPH between FABP and phospholipid bilayers, H-FABP or L-FABP were incubated with PADPH for 15 minutes and the fluorescence intensity measured. An equal volume of NBD-PE-containing vesicles was added, and the reaction mixture was incubated for 15 minutes. Since PADPH is quenched by the NBD fluorophore (> 98%), only FABP-bound PADPH remain fluorescent. Corrections for volume (50%) and NBD inner filter (always < 10%) were made, and the relative partition of PADPH between FABP and SUV (mol : mol) was calculated as follows:

$$\text{PADPH partition} = \frac{(\%\ \text{FABP-bound PADPH}/\mu\text{M FABP})}{(100 - \%\ \text{FABP-bound PADPH})\mu\text{M phospholipid}} \quad (1)$$

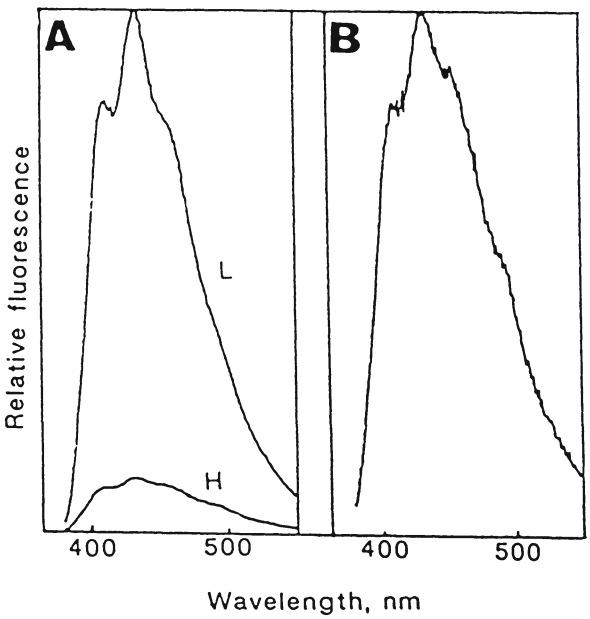

Fig. 1. Emission spectra of PADPH bound to FABP. Excitation was at 360 nm. A) 1 μM PADPH bound to 14 μM L-FABP (L) or H-FABP (H). Spectra are normalized to the L-FABP-bound PADPH maximum. B) PADPH bound to H-FABP, normalized to maximum intensity.

Results

Spectroscopic properties of PADPH bound to FABP

Fluorescence emission spectra of PADPH bound to H-FABP or L-FABP are shown in Fig. 1. The

intensity of L-FABP-bound fluorophore was markedly greater than that of an equal concentration of PADPH bound to H-FABP (Fig. 1A). Figure 1B shows the spectrum of PADPH bound to H-FABP normalized to its own maximum intensity. No spectral shift between PADPH bound to liver or heart FABP was seen, consistent with the lack of solvent effects on emission maxima of PADPH [6].

Excited state lifetimes (τ_F) of FABP-bound PADPH were measured by the phase modulation technique [13]. The results in Table 1 show that for both L- and H-FABP, PADPH lifetimes are >4 ns. These values are similar to those previously reported for PADPH in EPC membranes and organic solvents [5], and therefore indicate that the ffa binding sites of L-FABP and H-FABP are hydrophobic in nature. The quantum yield of PADPH fluorescence was found to be 0.50 for L-FABP- and 0.05 for H-FABP-bound ffa, reflecting the large difference in intensity seen in Fig. 1.

Although the τ_F data suggest a hydrophobic binding pocket on both proteins, the large difference in Q demonstrates that the L-FABP binding site is more hydrophobic than the H-FABP site. τ_F and Q values were large for L-FABP-bound PA DPH, and both of these parameters are known to increase as environmental polarity decreases [14, 15]. On the other hand, the long lifetime for H-FABP-bound PADPH combined with a low quantum yield suggests that depopulation of the PADPH excited state in H-FABP is largely via non-fluorescent decay. The results in Table 1 show that whereas for L-FABP-bound PADPH k_f and k_{nr} were equal, k_{nr} was 20-fold larger than k_f for H-FABP-bound PADPH.

Since the length of the PADPH fluorophore approximates that of a long chain ffa hydrocarbon chain, it is likely to be providing information about the entire ligand binding site [6]. This is in contrast to the AOffa analogues, where the fluorescent moiety is covalently attached at a series of specific positions along the length of the fatty acyl chain. Thus PADPH has the advantage of being a less 'perturbing' probe, whereas the AOffa allow information to be obtained at specific positions within the ffa binding site [12].

Table 1. Spectral properties of PADPH bound to FABP and EPC SUV. Data are for 1 or 2 μM PADPH, 10–14 μM FABP and 0.2 mM EPC. Modulation lifetimes are shown. Radiative and nonradiative decay rates (k_f and k_{nr}) were calculated from measured values of Q and τ_F using the equations $k_f = Q\tau_F$ and $\tau_F = 1/(k_f + k_{nr})$.

	L-FABP	H-FABP	EPC[a]
Quantum yield	0.50 ± 0.11	0.05 ± 0.02	0.73
Lifetime, ns	4.2 ± 0.6	4.0 ± 0.7	5.0
k_f, s^{-1}	1.20×10^8	0.12×10^8	1.45×10^8
k_{nr}, s^{-1}	1.20×10^8	2.36×10^8	0.53×10^8

[a] Trotter and Storch [6].

PADPH partition between FABP and membranes

PADPH was allowed to equilibrate between liver or heart FABP, and phospholipid vesicles. The relative distribution was assessed by comparing the PADPH fluorescence intensity in the absence and presence of NBD-PE-containing membranes, as described in Experimental procedures (equation 1). The results are expressed as the relative partition of PADPH between FABP and phospholipid, on a mol : mol basis. Table 2 shows that the molar partition preference of PADPH for L-FABP relative to EPC was 21 : 1, as compared to only 3 : 1 for H-FABP ($p < 0.005$). When studies were performed using SUV containing 25 mole% PS, a similar difference between the heart and liver proteins was observed. In addition, a comparison of PADPH partition between FABP and PC vs. PS-containing membranes, shows that the presence of bilayers with a net negative charge results in significantly

Table 2. Partitioning of PADPH between FABP and phospholipid membranes. The relative distribution of PADPH between FABP and SUV was calculated as described in Experimental procedures (equation 1). Results are expressed as the ratio of PADPH bound to FABP : phospholipid (mol : mol).

Phospholipid	L-FABP	H-FABP
PC	21.4 ± 1.6	3.3 ± 0.1[b]
PS	33.3 ± 1.4[a]	5.8 ± 1.0[a, b]

[a] $P < 0.025$ or lower, difference between PC and PS.
[b] $P < 0.005$ or lower, difference between L- and H-FABP.

greater partitioning of the ffa toward the FABP (Table 2).

2AP transfer from FABP to membranes

The transfer of 2AP from L-FABP or H-FABP to membranes containing the non-exchangeable fluorescence quencher NBD-PE was determined as described in Experimental procedures. Fig. 2 shows that ffa transfer is considerably faster from H-FABP as compared to L-FABP. The calculated rates were $0.59 \pm 0.11\,s^{-1}$ and $0.01 \pm 0.004\,s^{-1}$ for 2AP transfer from heart and liver FABP, respectively (mean ± S.D., n = 4).

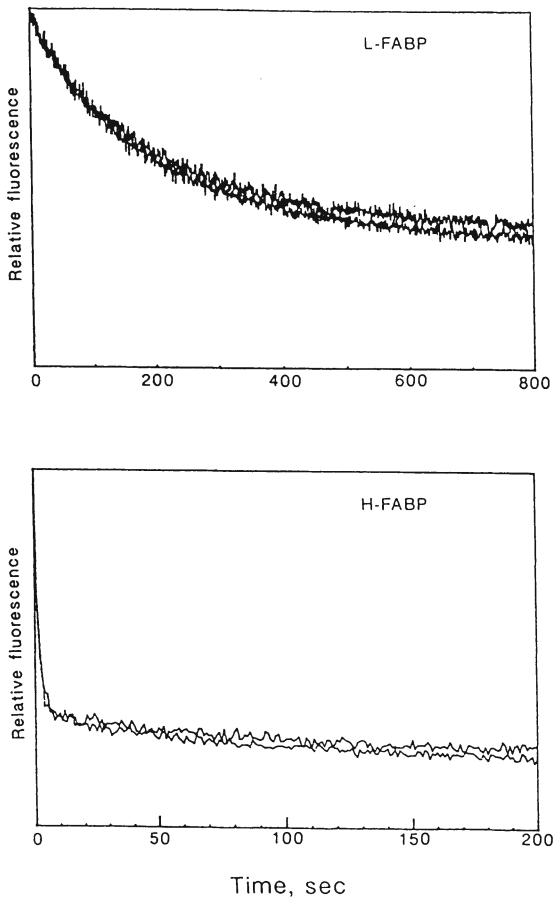

Fig. 2. 2AP transfer from FABP to EPC membranes. Transfer of 1.5 μM 2AP from 6 μM L-FABP or 5 μM H-FABP to 120 μM or 125 μM EPC SUV, respectively. Measurements were performed as described in Experimental procedures. Duplicate measurements are shown for each condition. Each time course was normalized to maximum intensity.

Discussion

Fluorescent analogues of ffa, including the parinaric acids and the anthroyloxy- and pyrene- derivatives, have been widely used to study ffa transfer kinetics in model membranes and cells, and to examine ffa binding to proteins [7, 14, 16–18]. Recent studies from this laboratory have shown that the negatively charged fluorophore PADPH is an excellent ffa analogue as well. It binds to rat L-FABP with a similar affinity and stoichiometry as native oleic acid, and adopts the same orientation in membranes as do native ffa [6]. The present studies have used the spectroscopic properties of PADPH to compare the physical nature of the ffa binding sites of L-FABP and H-FABP. In addition, PADPH and an anthroyloxy-ffa (AOffa) derivative were used to assess possible functional differences between these proteins by examining the relative partition of ffa between FABP and membranes, and the rate of ffa transfer from liver as compared to heart FABP.

The excited state lifetimes of PADPH suggest that both the L-FABP and H-FABP binding sites are relatively nonpolar. However, the lower quantum yield for H-FABP-bound PADPH indicates that the H-FABP site is more polar than the L-FABP binding site, in agreement with studies using the AOffa [12].[1] It was previously found that ffa are highly constrained within the binding site of L-FABP, but are markedly less constrained when bound to H-FABP [12].[1] Furthermore, whereas L-FABP-bound AOffa were not quenched by the aqueous phase quencher acrylamide [12, 18], substantial quenching of H-FABP-bound AOffa was observed.[1] The results suggest that the ffa binding site of H-FABP is more accessible to the aqueous phase and less hydrophobic than the L-FABP binding site.

The structural differences between heart and liver FABP may lead to functional differences be-

[1] Wooton, M.G., Bass, N.M., Bernlohr, D.A. and Storch, J., manuscript in press, Biochemistry.

tween the proteins. For example, the results of these studies demonstrate a marked difference in the abilities of L- and H-FABP to 'compete' with membranes for ffa. Whereas PADPH partition favors L-FABP by approximately 20-fold, in excellent agreement with the ^{13}C-NMR data of Cistola *et al.* [19], partition was only 3 : 1 for H-FABP: phospholipid. In support of this comparatively low distribution of PADPH toward the heart FABP, Glatz *et al.* have recently reported that little ffa is released from damage myocardium despite the release of large amounts of H-FABP [20].

Moreover, Reers *et al.* have previously shown that when mixtures of AOffa, PC vesicles and either L- or H-FABP were separated by gel filtration, most of the AOffa fluorescence was associated with L-FABP, but in the case of H-FABP, most was associated with the membrane vesicles [21]. We have obtained similar results with the AOffa,[2] using the vesicle quenching assay used for the PADPH studies.

The observed 6-fold difference in relative PA DPH partitioning between L- or H-FABP and membranes could be due to differences in either the binding affinity or capacity between the two proteins. It is generally thought that the affinities of L- and H-FABP for ffa are similar, although some studies suggest a slightly higher affinity for the liver protein [24–27]. The stoichiometry of ffa binding to heart and liver FABP is also thought to be similar, although somewhat higher binding capacities for L-FABP have been shown [25, 26]. Both of these could contribute in part to the partitioning differences observed here. In addition, the observed difference could be caused by the analogue nature of PADPH, which may not reflect the native ffa in a quantitative manner.

The present results show, in agreement with previous reports [21–23], that net transfer of ffa from FABP to membranes occurs. The present studies also demonstrate a striking difference in the rate of transfer of 2AP from FABP to membrane vesicles, with transfer from the heart protein >50-fold more rapid than from the liver protein. Since ffa transfer is likely to occur via diffusion through the aqueous phase rather than by collision of the FABP with membranes [7],[2] the observed transfer rates presumably reflect the dissociation or off-rate of the ffa from the protein. Thus the less hydrophobic and less constrained binding site of H-FABP may contribute both to its lower ffa partition preference vs. membranes, and the faster off-rate of ffa. The large differences in ffa transfer rates and patitioning between L- and H-FABP suggest that their functions in cellular ffa transport and distribution may be unique. It is possible that L-FABP acts as an intracellular storage pool for ffa, whereas H-FABP may serve to rapidly release ffa for myocardial oxidation. However, the high degree of homology of H-FABP with the adipocyte FABP makes it likely that FABP function is not solely related to the level of cellular ffa oxidation.

The question of whether fatty acid-binding proteins alter the rate of ffa transport between cellular membranes is beginning to be addressed [22, 23]. Rapid spontaneous transfer rates of ffa make it difficult to obtain kinetic data when donor and acceptor membranes must by physically separated [22, 23]. In the present studies, the fluorescence energy transfer system obviates the need for separating donor and acceptor membranes, and accurate kinetic data is obtained. Although the absolute results must be viewed in terms of the analogue nature of these fluorophores, relative rates are likely to be valid. This approach should prove useful for defining the role of FABP in ffa traffic within the cell, and for assessing whether different FABP have distinct functions in cellular ffa distribution.

Acknowledgements

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Role of fatty acid-binding protein in cardiac fatty acid oxidation

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Key words: FABP, fatty acid, cardiac energy

Summary

Although abundant in most biological tissues and chemically well characterized, the fatty acid-binding protein (FABP) was until recently in search of a function. Because of its strong affinity for long chain fatty acids and its cytoplasmic origin, this protein was repeatedly claimed in the literature to be the transcytoplasmic fatty acid carrier. However, techniques to visualize and quantify the movements of molecules in the cytoplasm are still in their infancy. Consequently the carrier function of FABP remains somewhat speculative. However, FABP binds not only fatty acids but also their CoA and carnitine derivatives, two typical molecules of mitochondrial origin. Moreover, it has been demonstrated and confirmed that FABP is not exclusively cytoplasmic, but also mitochondrial. A function for FABP in the mitochondrial metabolism of fatty acids plus CoA and carnitine derivatives would therefore be anticpated. Using spin-labelling techniques, we present here evidence that FABP is a powerful regulator of acylcarnitine flux entering the mitochondrial β-oxidative system. In this perspective FABP appears to be an active link between the cytoplasm and the mitochondria, regulating the energy made available to the cell. This active participation of FABP is shown to be the consequence of its gradient-like distribution in the cardiac cell, and also of the coexistence of multispecies of this protein produced by self-aggregation.

Introduction

Long-chain fatty acids, which are catabolized by the mitochondrial β-oxidative system, are considered as the major fuel of energy production in the heart [1–4]. The accepted general consensus states that acylchains are translocated from the cytoplasm into the mitochondria, then β-oxidized in the mitochondrial matrix, yielding acetyl-CoA to the Krebs cycle and $NADH_2$ plus $FADH_2$ to the respiratory chain and finally ATP by oxidative phosphorylation.

A few years ago, a major point was brought up [5–7]: what mechanisms regulate the energy output of this fatty acid-dependent system, so as to match the frequent boosts of energy required by the heart?

Studies with the isolated perfused rat heart, supplemented with palmitate, have shown [5] that, when the energy requirement was low (low ventricular pressure, 60 mmHg, and thus low rate of oxidative phosphorylation) palmitate-derived acetyl CoA accumulated, as if its entrance into the Krebs cycle was the limiting step of energy production. However, when the cardiac energy requirement was high (high ventricular pressure, 120 mmHg, and thus high rate of oxidative phosphorylation) the accumulating species was acylcarnitine, suggesting that the translocation of acylcarnitine through the mitochondrial membranes is the limiting step of cardiac energy production [5].

The mechanisms behind this acylcarnitine-dependent energy regulation have not yet been investigated. Any explicative model should take into account the possible participation of the following four elements which are linked to the acylcarnitine

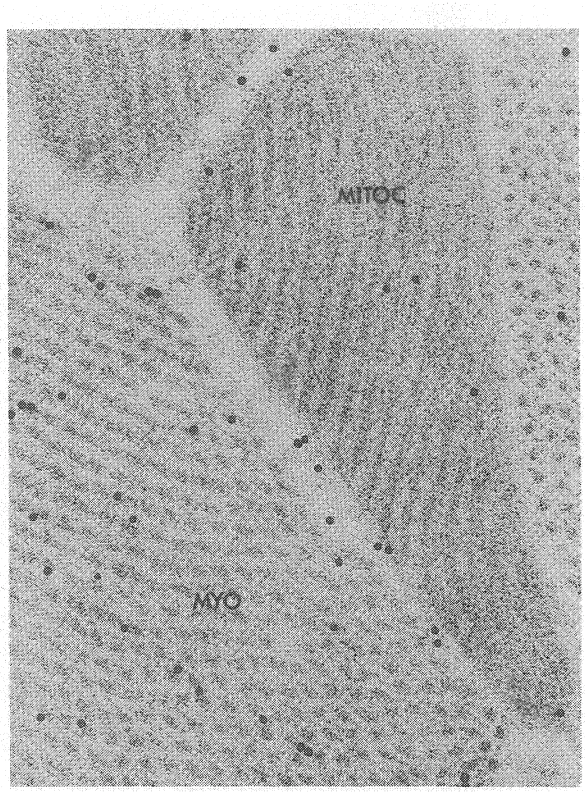

Fig. 1. Subcellular detection of FABP in rat heart by the immunogold method. The procedure is detailed in ref. 9. MYO, myofibrils; MITOC, mitochondria.

metabolism in the mitochondria: 1. The acylcarnitine transferase I, bound to the outher surface of the inner mitochondrial membrane, and controlling the transformation of acylCoA into acylcarnitine. 2. The acylcarnitine translocase, bound to the inner mitochondrial membrane, and controlling the shuttling of acylcarnitine through the inner mitochondrial membrane. 3. The acylcarnitine transferase II, bound to the inner surface of the inner mitochondrial membrane, and controlling the transformation of acylcarnitine into acylCoA. as a preliminary step before acylCoA β-oxidation in the mitochondrial matrix. 4. The fatty acid-binding protein (FABP), localized in all cell compartments, including mitochondria [9], and able to bind not only fatty acids and acylCoA but also acylcarnitine [8].

Evidence will be presented here, showing that FABP binding and interactions with acylcarnitine, and the resulting control exerted by FABP on the acylcarnitine translocation into the mitochondria, might be the central element behind this acylcarnitine-dependent energy production in the cardiac muscle.

Experimental procedures

FABP purification, measurement and localization; mitochondria isolation; β-oxidation rate measurements; fatty acid, CoA and carnitine derivative quantitation; electron spin resonance (ESR) and computer procedures; 16-doxylstearoyl carnitine synthesis, as described in references [8–9]. Mathematical background and determination of parameters required for the modelling are described in reference [10].

Synthesis of 16-doxyl Stearoyl CoA

As a first step, coenzyme A (Boehringer) was purified by chromatography on Kieselgel$_{60}$ – F_{254} – 0,25 mm – plates (Merck). The solvent was Chloroform – methanol – water (45 : 45 : 10, by vol). The major band detected under UV (254 nm) was scratched, extracted with water, and finally lyophilized.

Fifty μmoles 16-doxylstearic acid (Aldrich) were mixed for 60 min with 60 μmoles 1,1′-carbonydiimidazol in 2 ml tetrahydrofuran. After evaporation under N_2, the sample was solubilized in 1 ml tetrahydrofuran-water (2: 1, v/v), then mixed with 55 μmoles of the purified CoA solubilized in 5 ml tetrahydrofuran-water (2 : 1, v/v). After setting the pH between 7 and 7,5 with 0,5 N NaOH, the solution was stirred overnight at room temperature, under N_2, and then lyophilized. The yield was about 60 mg and was divided in two portions of 30 mg.

The next step was the purification on Sep-Pack C_{18} Cartridge (Waters). The micro-column was washed with 2 ml methanol, then 5 ml water. The above 30 mg were solubilized in 4 ml (methanol-water, 5: 5), then applied on the column. The filtrate was recycled 10 times through the column.

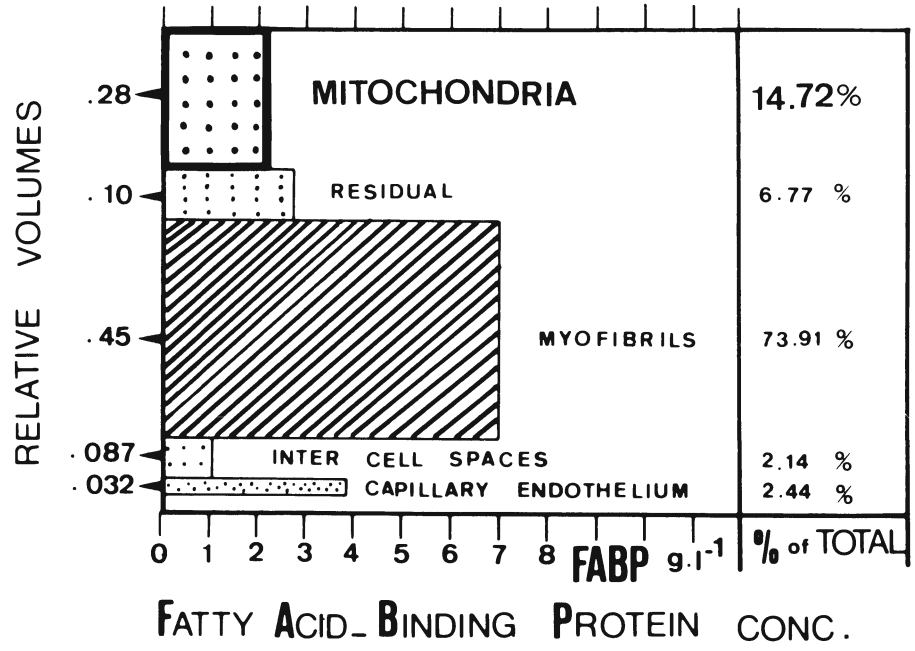

Fig. 2. Subcellular distribution of FABP in rat heart. The techniqaue is described in ref. 9

Finally the adsorbed compounds were eluted with 30 ml volume of the following successive solvents: (A) water; (B) methanol: water v/v, 1 : 9; (C) 2 : 8; (D) 3 : 7; (E) 4 ; 6; (F) 5 : 5; (G) 6 : 4; (H) 7 : 3; (I) 8 : 2; (J) 9 : 1; (K) methanol.

The B, C, D, E fractions containing partially purified acylCoA, plus residual fatty acid, were pooled and lyophilized.

After solubilization in 2 ml methanol, a biphasic system was obtained by adding 5 ml hexane and shaking. This washing procedure was repeated 6 times. The final methanolic phase was evaporated and the residue solubilized in 3 ml n-butanol. Four ml water saturated with n-butanol were added. The solution was shaken and left overnight at 4° C. The butanolic upper phase was collected and mixed with 4 ml water saturated with n-butanol. After 1 h at 4° C, the sample was centrifuged 5 min at 350 g. The water containing phase was collected and contained pure 16-doxylstearoyl CoA, as confirmed by TLC (single spot), ESR (typical nitroxide radical triplet) and spectrophotometry (characteristic CoA peak between 230–300 nm.

Results

The subcellular localization of FABP was determined by using specific FABP polyclonal antibodies conjugated to small (19 mm) gold particles (Fig. 1). Most of FABP (73,9% was found on the myofibrils (Fig. 2). However, remarkably 14,7% was detected in the mitochondria (Figs 1–2). A strong FABP concentration gradient delineates the myofibrils (6.9 mg · ml^{-1}) from the mitochondria (2.2 mg · ml^{-1}).

Although named fatty acid-binding protein, FABP also binds acylCoA and acylcarnitine, two typical derivatives synthetized by the mitochondria in the cardiac muscle. This is readily shown by the electron spin resonance technique (ESR) applied to 16-doxylstearic acid, stearoyl CoA and stearoylcarnitine, interacting with FABP (Fig. 3). In the presence of FABP the ESR signal of these three compounds (Fig. 3) is enlarged and the amplitudes of the peaks decrease. This is the typical behaviour of a nitroxide-labelled molecule when its motion is restricted by binding to a macromolecule.

Because of its mitochondrial localization and the capacity to bind typical acylchains normally pro-

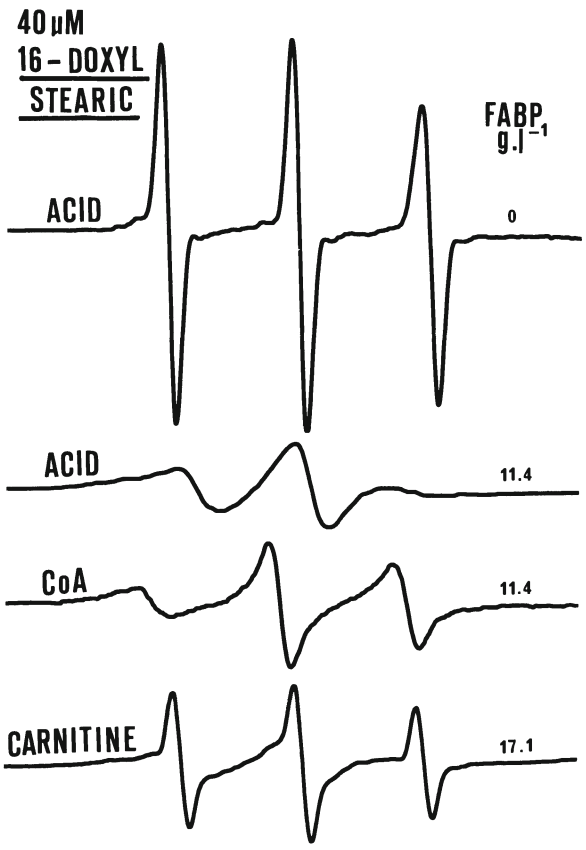

Fig. 3. Binding of fatty acid, acylCoA and acylcarnitine to FABP. Purified FABP from rat heart, as described in ref. 9, was mixed in 50 µl glass capillaries with 16-doxylstearate (K salt), 16-doxylstearoylCoA or 16-doxylstearoylcarnitine, in a buffer containing 7.9×10^{-3} M sodium phosphate (pH 7.4), 3.97×10^{-4} M EDTA, 6.7×10^{-2} M KCl and 0.1 M sucrose. The electron spin resonance signals were recorded at 37° C on a Varian E line spectrometer (X band) using 100-kHz field modulation.

cessed by the mitochondria, such as fatty acids, acylCoA and acylcarnitine, an important function for the mitochondrial FABP can be anticipated.

To investigate this hypothesis, we isolated intact (Fig. 4) and biochemically coupled mitochondria [8] from rat heart. Using [1-^{14}C] stearate as a precursor and FABP as a carrier, the mitochondrial β-oxidative output was analyzed, as a function of variable FABP concentrations (Fig. 5). The β-oxidation profile is quite dependent upon the FABP concentration. When FABP increases, the β-oxidation output is exponentially decreasing. However, very remarkably, a strong boost of activity is observed around $2\,g \cdot l^{-1}$ (Fig. 5).

We speculated [9] that such a profile could be the consequence of the self-aggregated nature of FABP. One of the FABP isoforms might be more competent than the others in controlling the β-oxidative system. In fact, the theoretical model which was partially developed elsewhere [10] and detailed later herein, predicts such a boost of activity when considering an enzymatic system where a self-aggregated protein is the carrier providing the substrate.

In this model (Fig. 6), we studied the activity(R) of an enzyme bound to a membrane (M), when the substrate (S) could be supplied to the enzyme by a self-aggregated protein (P). What is the influence of the self-aggregation capacity of the protein on the final activity of the enzyme?

This model was applied here to the mitochondrial β-oxidative system when the substrate supplier was considered to be FABP, a self-aggregated protein. In an equilibrium system containing isolated mitochondria, FABP and fatty acid, the latter is expected to partition between the aqueous medium (A_s), the mitochondrial membranes (A_{in}) and bound (A_b) to FABP (Fig. 6).

To find the dependence of the β-oxidative activity R upon the self-aggregated FABP, the following set of equations is required:

$$R = \frac{R_{max}\,[S]}{K_m + [S]} \qquad (1)$$

This equation assumes that the dependence of the enzyme activity R on the fatty acid substrate concentration [S] is of the Michaelis-Menten type. The following arbitrarily chosen values have been assigned:

$$R_{max} = 5.08 \times 10^{-4}\,M \cdot min^{-1}\ (g\ of\ protein)^{-1},$$
$$K_m = 2.5 \times 10^{-4}\,M.$$

Hence:

$$R = \frac{5.08 \times 10^{-4}\,[S]}{2.5 \times 10^{-4} + [S]} \qquad (2)$$

The three following sources of [S] were considered:

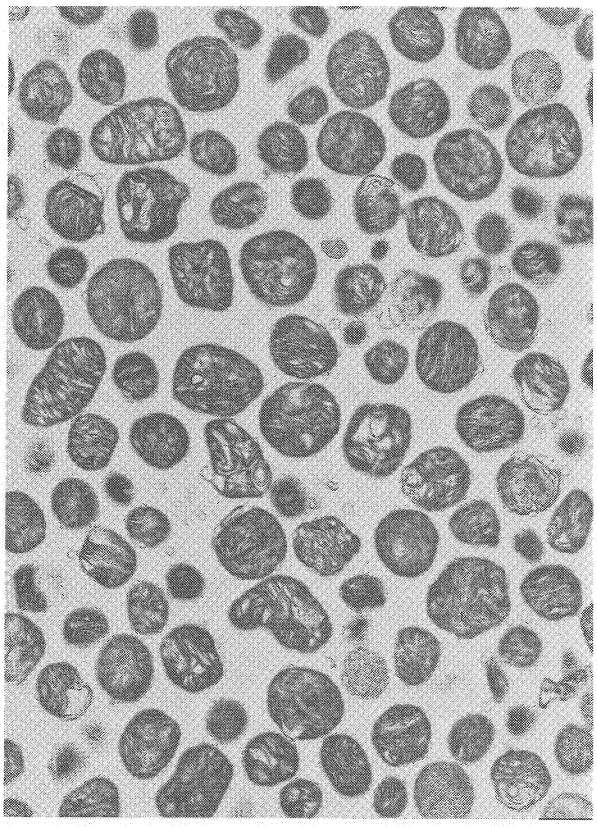

Fig. 4. Electron microscopy of isolated mitochondria from rat heart. Isolation procedure as described in ref. 9. After fixation in 2% glutaraldehyde and post-fixation in 1% O_SO_4, the pellets were embedded in EPON. The sections were contrasted for 1 min. with lead citrate according to Reynolds [11]. Enlargement 10400 ×.

a) A_S is the source of substrate

This means that the substrate for the β-oxidative enzymes is assumed to be the fatty acid which is free in the aqueous medium, and reaches the enzymes by simple diffussion.

Before substituting (S) by (A_s) in eq, 2, an explicit value of (A_s) as a function of the carrier (P) is required. The following set of equations delineate the final expression required: $[A_s] = f[P]$.

$$[A_t] = [A_{in}] + [A_S] + [A_b] \quad (3)$$

$$[A_S] = [A_t] - [A_{in}] - [A_b] \quad (4)$$

Equations 3 and 4 state that the total fatty acid $[A_t]$ considered, is distributed between the mitochondrial membrane $[A_{in}]$, free in the medium $[A_S]$, and bound $[A_b]$ to the carrier [P].

$[A_t]$ was arbitrarily set equal to 1×10^{-4} M.

$$[A_S] = 10^{-4} - [A_{in}] - [A_b] \quad (5)$$

To define $[A_S]$, explicit values of $[A_{in}]$ and $[A_b]$, as a function of [P] remain to be determined.

$$[A_b]/[P] = v \quad (6)$$

$$[A_b] = v\,[P] \quad (7)$$

Equations 6 and 7 describe the binding isotherm v, of fatty acid to the carrier (P). v is defined by the following phenomenological equation, explicited in ref (10):

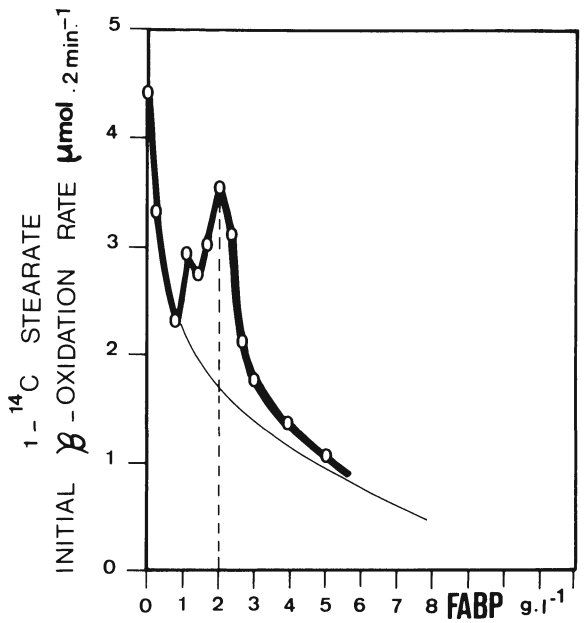

Fig. 5. Control by FABP of fatty acid β-oxidation in isolated rat heart mitochondria. According to the method described in ref. 9.

$$v = \frac{V_{maxI}}{1 + K_I/[A_S]} + \frac{V_{maxII}}{1 + (K_{II}/[A_S])^{nII}} + \frac{V_{maxIII}}{1 + (K_{III}/[A_S]^{nIII}} \quad (8)$$

Nummerical values of parameters K_i and n_i and v_{max} can be found in ref [10].

$$[A_{in}]/[A_S] = p \cdot [M] \quad (9)$$

$$[A_{in}] = p \cdot [M]\,[A_S] \quad (10)$$

Equations 9 and 10 define the partition coefficient, p, of fatty acid between the aqueous medium (A_S) and the membrane (A_{in}) of the mitochondria (M). We experimentally observed that the ratio $[A_{in}]/[A_S]$ varied linearly with [M] and the partition coefficient, p, was calculated to be $3.55\,1 \cdot (g \cdot prot)_-^{1}$.

$$[A_{in}] = 3.55\,[M]\,[A_S] \quad (11)$$

Substitution of $[A_b]$ and $[A_{in}]$ in equation 5, by their respective values defined in equations 7 and 11 gives rise to:

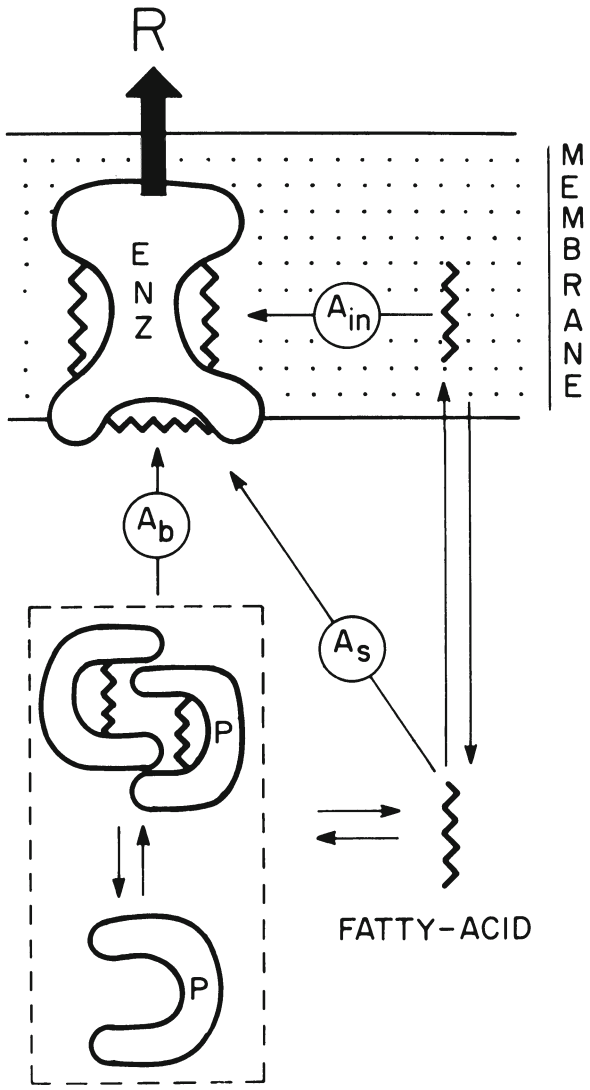

Fig. 6. Model for the interactions of a self-aggregated protein (P) with fatty acids and with a membrane-bound enzyme using as substrate fatty acids issued from the medium (A_S), from the membrane (A_{in}), or from the binding sites (A_b) on the protein.

$$[A_S] = (10^{-4} - v\,[P])/(1 + 3.55\,[M]) \quad (13)$$

This explicit value of $[A_S]$ as a function of [P], is then used for [S] in equation 2:

$$R = \frac{5.08 \times 10^{-4}\,(10^{-4} - v\,[P])/(1 + 3.55\,[M])}{2.5 \times 10^{-4} + (10^{-4} - v\,[P])/(1 + 3.55\,[M])} \quad (14)$$

Using the numerical values of ref [10] to explicit v,

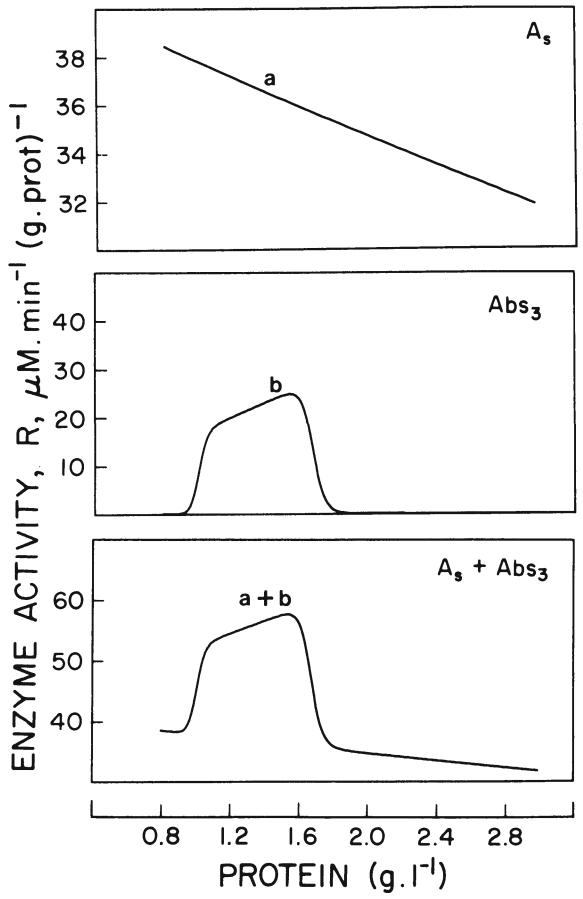

Fig. 7. Computer simulation of the enzyme activity (R) in the model of Fig. 6. The substrate for the enzyme is the freely diffusing fatty acids (A_S), or the fatty acid (A_b) specifically bound to S_3, one of the different self-aggregated species of the protein.

and giving the arbitrary value $1\,(g \cdot prot)^{-1}$ to [M], the activity R of the enzyme can be computer calculated as a function of FABP concentrations, [P], when the fatty acid supplied to the β-oxidative enzymes is diffusing from the aqueous medium.

Increasing the concentration of FABP, from 0.8 to $2.8\,g \cdot l^{-1}$, induces and exponential decrease of the enzyme activity (Fig. 7a).

b) The substrate S is delivered to the β-oxidative enzymes only by A_{bS3}, one of the self-aggregated FABP species

Fatty acid bound, (A_b), to the different FABP species, S_1, S_2, S_3, S_4, are respectively labelled: A_{bS1}. A_{bS2}, A_{bS3}, A_{bS4}. We assume that only A_{bS3} can be a substrate for the β-oxidative enzymes. When the total concentration [P] of FABP is varied, the equilibrium concentrations of the different self-aggregated species S_1–S_4, are expected to vary, and the quantity of substrate selectively delivered by the species A_{bS3} to the enzymes must fluctuate considerably. Consequently, the activity of the enzymatic system should also fluctuate.

This idea can be made objective according to the following rationale.

$$[A_b] = v\,[P] = v\,([S_1] + [S_2] + [S_3] + [S_4]) \quad (15)$$

$$[A_{bS3}] = v\,[S_3] \quad (16)$$

Equation 15 characterizes the binding isotherm of fatty acid to FABP, and equation 16 the binding isotherm of fatty acid A_{bS3} to S_3, one of the self-aggregated FABP species.

To define $[A_{bS3}]$ as a function of [P], explicit values of v and $[S_3]$ must be found as a function of [P] in equation 16.

1) Explicitation of v

$$[A_t] = [A_{in}] + [A_S] + [A_b] \quad (17)$$

From equations 7 and 10, we can write:

$$[A_t] = p \cdot [A_S]\,[M] + [A_S] + [P]\left(\frac{V_{maxI}}{1 + K_I/[A_S]} + \frac{V_{maxII}}{1 + (K_{II}/[A_S])^{nII}} + \frac{V_{maxIII}}{1 + (K_{III}/[A_S]^{nIII}}\right) \quad (18)$$

As stated previously we arbitrarily attributed the following values: $[A_t] = 1 \times 10^{-4}$ M; $[M] = 1\,(g \cdot prot)^{-1}$, and p was calculated to be equal to $3.55\,1(g \cdot prot)^{-1}$. The numerical values of parameters v_{max}, K_i and n_i, can be found in reference [10].

Finally, the explicit values of v as a function of [P], are obtained by extracting $[A_S]$ from eq. 18 and then subtituting it in eq. 8.

2) Explicitation of $[S_3]$

According to our previous studies [10], explicit values of $[S_3]$ as a function of [P] are defined by:

$$[S_3] = \frac{[P]\,[P]^{n3+n2}}{a_3^{n3}a_2^{n2}\cdot F} \qquad (19)$$

$$F = 1 + \frac{[P]^{n3}}{a_3^{n3}} + \frac{[P]^{n3+n2}}{a_3^{n3}a_2^{n2}} + \frac{[P]^{n3+n2+n1}}{a_3^{n3}a_2^{n2}a_1^{n1}} \qquad (20)$$

The significance and the numerical values of parameters n_i and a_i, can be found in Table I of ref. [10].

Explicitation of v and $[S_3]$ as a function of [P] is thus achieved. After substitution of v and $[S_3]$ by their explicit values in eq. 16, the required relation: $[A_{bS3}] = f[P]$ is obtained.

Finally, the activity, R, of the β-oxidative enzymes can be computer calculated as a function of [P], the total FABP concentration, by substituting [S] in eq. 2 by the explicit values $[A_{bS3}] = f[P]$ defined above.

Increasing the total FABP concentration, [P], from 0.8 to 2.8 g · l^{-1}, when the fatty acid is translocated towards the enzymes only by S_3, one of the self-aggregated FABP species, induces a boost of activity as shown graphically in Fig. 7b.

c) The substrate S, is delivered to the β-oxidative enzymes, concomitantly by A_S and by A_{bS3}

The explicited values of A_S and A_{bS3}, as defined previously, are concomitantly set for [S] in eq. 2. The resulting enzyme activity R, when varying [P], is described by an exponential decreasing function, but remarkably modulated by an important boost of activity in the middel of the curve (Fig. 7a + b).

The general profile as described by this theoretical model (Fig. 7), is in good agreement with the experimental results (Fig. 5). The boost of β-oxidative activity experimentaly observed around 2 g · l^{-1} (Fig. 5) is the consequence of the self-aggregated nature of FABP. In this perspective,

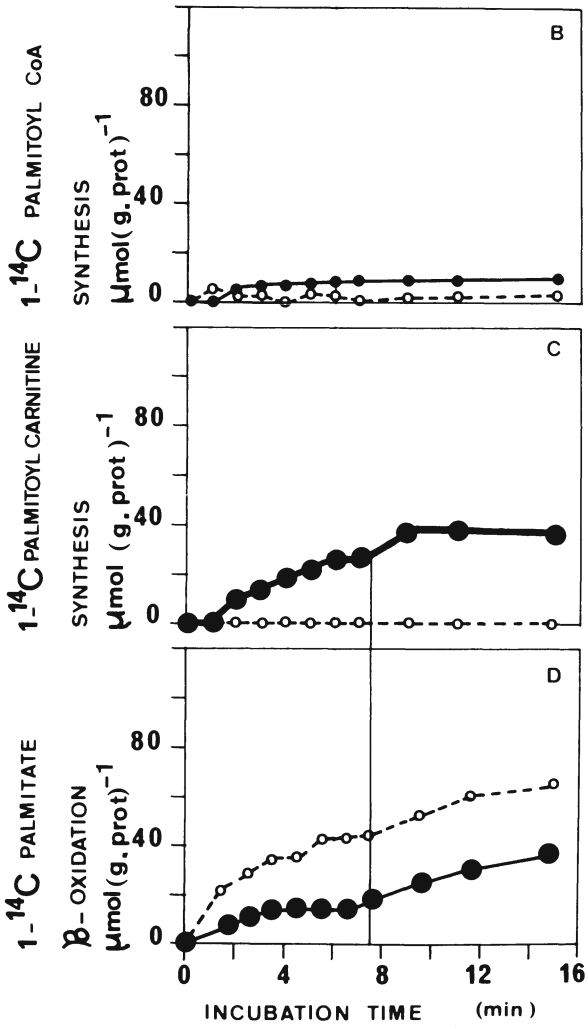

Fig. 8. β-oxidation of palmitate (D) by isolated rat heart mitochondria in the absence (---) or in the presence (——) of FABP (2 g · l^{-1}), and concomitant formation of palmitoylcarnitine (C) and palmitoylCoA (B). The technique is described in ref. 8.

this original property of FABP, appears as a powerful tool to regulate the cardiac energy production. Only slight modifications of FABP concentrations in the mitochondrial environment are required to modulate the energy output.

The next stage of this study was to delineate which step of this complex mitochondrial β-oxidative system is directly FABP-dependent. The pertinence of the question is clear, if we consider that FABP can bind the three major substrates of

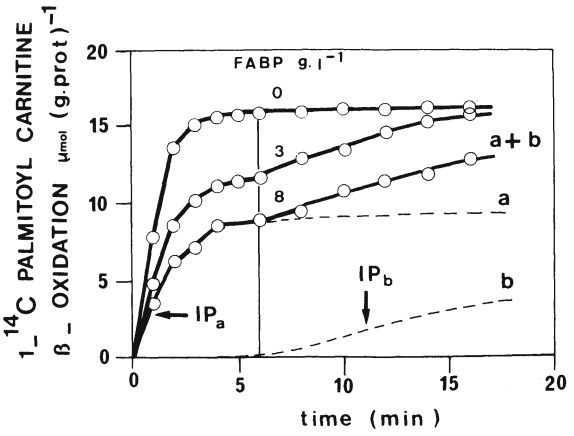

Fig. 9. Influence of FABP on the kinetics of palmitoylcarnitine β-oxidation by isolated rat heart mitochondria. Each kinetic was deconvoluted into two sigmoidal curves, a, b, with their respective inflection point IP_a, IP_b, where the maximal β-oxidation rate can be calculated. Procedures as described in ref. 8.

this enzymatic system: fatty acid, acylCoA and acylcarnitine (Fig. 3).

Using [1-^{14}C] palmitate as a precursor and FABP as a carrier, the mitochondrial production of palmitoylCoA, palmitoylcarnitine, and their subsequent β-oxidation, was compared to the situation where FABP was omitted.

In the absence of FABP, [1-^{14}C] palmitate is rapidly β-oxidized (Fig. 8D). No accumulation of palmitoylCoA (Fig. 8B) or palmitoycarnitine (Fig. 8C) is observed. The presence of FABP ($2\,g \cdot l^{-1}$) strongly depressed the β-oxidation output (Fig. 8D); concomitantly acylcarnitine accumulates (Fig. 8C) as if the accessibility of acylcarnitine to the β-oxidative system was restricted by FABP. This is confirmed in Fig. 9, where [1-^{14}C] palmitoylcarnitine β-oxidation kinetics, are shown to be strongly FABP-dependent. These kinetics develop in two succesive phases (Fig. 9). The first one reaches a zero rate value after about 6 min and the second phase starts after about 8 min and shows a quasi-linear evolution. All these kinetics could be deconvoluted into two sigmoidal curves, respectively characterized by their inflection points, IP_a, IP_b (Fig. 9). The first phase (< 6 min) is correlated with the decay of A_S, the acylcarnitine from the aqueous medium (Fig. 10). The second phase

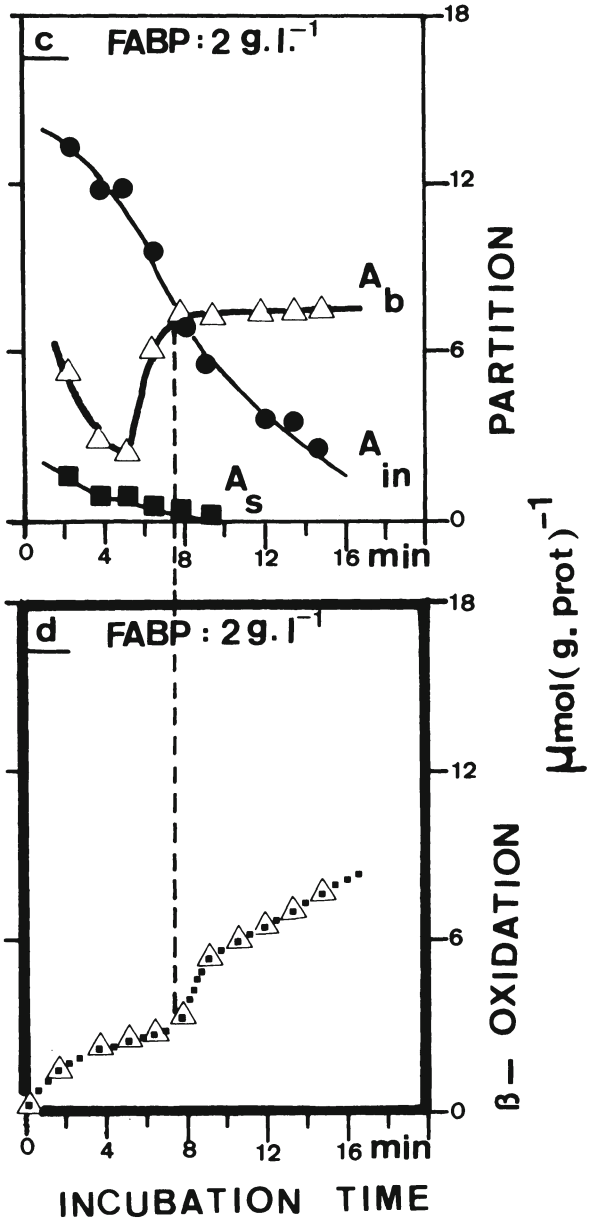

Fig. 10. Analysis by electron spin resonance of β-oxidation kinetics of the stearic acid analog 16-doxylstearoylcarnitine by isolated rat heart mitochondria and concomitant partition in the mitochondrial membrane (A_{in}), free in the medium (A_S) and bound (A_b) to FABP. Procedures as described in ref. 8.

(> 8 min) is correlated with A_b, the acylcarnitine bound to FABP (Fig. 10). This means that acylcarnitine can reach the β-oxidative machinery by two pathways: first, by direct diffusion from the aqueous medium (A_S), and second, via the media-

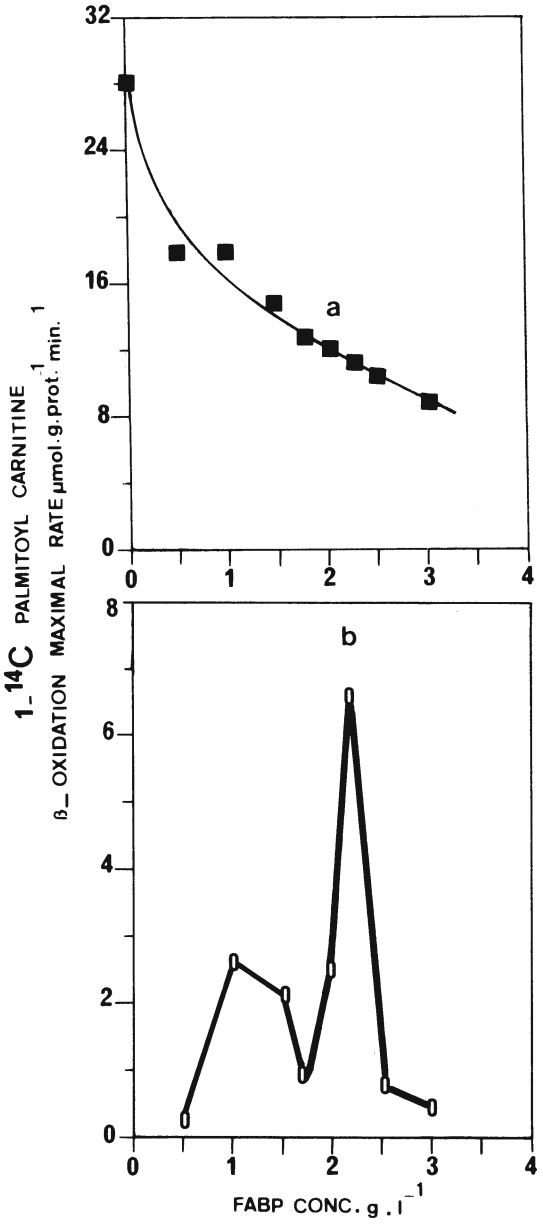

Fig. 11. Influence of FABP on the maximal β-oxidation rate of [1-^{14}] palmitoylcarnitine in isolated rat heart mitochondria, when palmitoylcarnitine is issued from the aqueous medium (a) or from the binding sites on FABP (b). The rates were calculated from the inflection point IP as described in Fig. 9 and according to the procedure detailed in ref. 8.

tion of FABP which can give up its bound acylcarnitine, (A_b), to the β-oxidative system.

The maximal rates of both pathways were determined by calculating the derivatives at the inflection points, IP_a, IP_b, of the deconvoluted sigmoidal curves (Fig. 9). A remarkable result emerges (Fig. 11b). In the physiological range of mitochondrial FABP concentration observed in vivo (Fig. 2), i.e. around $2\,g \cdot l^{-1}$, the in vitro FABP-dependent β-oxidation shows a boost of activity, (Fig. 11b), as predicted by the theoretical model described before (Fig. 7), when only one of the different FABP self-aggregated species was assumed to translocate fatty acid to the β-oxidative system.

Furthermore, the β-oxidation rate of acylcarnitine, A_S, not translocated by FABP, but freely diffusing, is also in agreement with the theoretical model described before (Fig. 7a). An exponential decrease in activity is observed when FABP concentration increases (Fig. 11a). The reason is obvious if we consider that the A_S pool must decrease if part of its acylcarnitine binds to FABP when the concentration of the latter increases.

In summary, the two types of acylcarnitine involved in the β-oxidative system are strongly dependent upon FABP.

Conclusions

The *in vivo* limiting step of energy production, when the heart requires high energy output, was suggested to be the transport of acylcarnitine into the mitochondria [5]. As far as we know, no mechanism has been proposed to explain this important regulation.

The present study delineates FABP and its self-aggregation capacity as a possible parameter to be considered in this acylcarnitine-dependent energy production. Acylcarnitine translocation into the mitochondria is shown to be mediated by this protein. An optimal mitochondrial energy output is obtained when the FABP concentration is around $2\,g \cdot l^{-1}$. According to the theoretical model developed here such an optimalization is the consequence of the participation of only one of the self-aggregated FABP species translocating acylcarnitine. The driving force which could generate this competent translocator is the local FABP concentration. Any perturbation of the FABP gradient between the myofibrilar cytoplasm ($6.9\,g \cdot l^{-1}$) and

the mitochondria ($2.2\,g \cdot l^{-1}$), is expected to induce concentration changes of this acylcarnitine competent translocator, and thus might induce important variations of the cardiac energy production.

Future research, dedicated to the physiological or pathological factors able to maintain or to disrupt respectively, this in vivo optimalized FABP gradient, are promising when considering the regulation of the cardiac energy. High mechanical activity of the heart muscle might disturb this optimalized FABP gradient and consequently may limit acylcarnitine access to the β-oxidative system.

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Modulation of fatty acid-binding capacity of heart fatty acid-binding protein by oxygen – derived free radicals

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Key words: heart, fatty acid-binding protein, free radicals

Summary

In this study, we examined the effects of exposure of heart fatty acid-binding protein (h-FABP) to chemically generated O_2^- or OH · with respect to its oleate binding and to its electrophoretic properties. Purified rat h-FABP at 40 μM scavenged as much as 30% O_2^- and 85% OH ·. On the other hand, when 2 nmol (4 μM) FABP were exposed to free radicals, the maximum oleate binding capacity as measured by Scatchard analysis was reduced only by 14% and 27% for O_2^- and OH ·, respectively. The electrophoretic pattern of free radical-exposed FABP was not markedly different when examined either by the non-denaturing or by denaturing PAGE, suggesting the absence of any degradation or aggregation of FABP by O_2^- or OH ·. It is hypothesized that O_2^- or OH · in physiological concentration may not alter the function of FABP markedly in the ischemic-reperfused myocardium.

Abbreviations: h-FABP – Heart Fatty Acid Binding Protein, NEFA – Non-Esterified Fatty Acids, O_2^- – Superoxide anions, OH · – hydroxyl radicals, OCI · – hypohalite radicals, H_2O_2 – hydrogen peroxide, HPLC – High Pressure Liquid Chromatography

Introduction

Fatty acid-binding protein (FABP) is a major cytosolic protein present in intestinal mucosa, liver, and myocardium [1, 2]. These proteins exhibit high binding affinities for long chain non-esterified fatty acid (NEFA), and their acyl-CoA derivatives [3]. By virtue of these properties and its ability to affect several metabolic pathways, FABP has been implicated as an intracellular transport protein of NEFA, [4] an attenuator of acyl-CoA and NEFA mediated inactivation of enzyme systems as well as a positive modulator of phospholipid synthesis [5].

The reperfusion of ischemic myocardium is associated with the generation of free radicals such as superoxide anions (O_2^-), hydroxyl radicals (OH ·), hypohalite radicals (OCI ·), and oxidants such as hydrogen peroxide (H_2O_2) [6]. Free radical mediated cellular injury appears to result from the loss of membrane function as a consequence of degradation of phospholipids, lipid peroxidation and the inactivation of proteins [7, 8]. The reperfusion of ischemic myocardium is also associated with the accumulation of NEFA and their thioesters, which are known to cause arrythmia and myocardial dysfunction [9]. FABP, being in intracellular protein, is likely to play a significant role in the ischemic heart because of its ability to bind NEFA and their thioesters with high affinity. This study was undertaken to examine whether the exposure of FABP to oxygen-derived free radicals results in a) alteration of its binding characteristics and b) modification of

its protein structure. The results presented in this paper suggest that the exposure of FABP to physiological concentrations of O_2^- or OH · may not alter its fatty acid binding ability markedly.

Experimental procedures

FABP preparation
Rat heart cytosolic FABP was prepared essentially according to the method of Ockner *et al.* [4] as described previously [10]. In brief, the method involved the gel filtration of myocardial cytosol on Sephadex G-75 column, followed by DEAE-cellulose chromatography of the low molecular weight fraction of the gel filtration eluate. The fraction containing FABP was concentrated by ultrafiltration using UM-5 membrane (Whatman) which was subjected to a second gel filtration on a Sephadex G-75 column. The isolated FABP was found to be nearly homogenous as judged by 15% SDS-PAGE and had an apparent molecular weight of 14,000 D.

Delipidation of FABP
The FABP was subjected to dilipidation using Lipidex 1000 as described by Glatz *et al.* [11]. 2 mg of FABP was loaded onto a column (0.5 × 5 cm) of Lipidex 1000 equilibrated with 100 mM Tris-HCL buffer, pH 7.4, containing 1 mM dithiothreitol at 37° C. The dilipidated FABP emerged in the void volume and its oleate binding capacity was determined essentially according to the procedure described by Offner *et al.* [12].

Generation of free radicals
The O_2^- was generated by the action of xanthine oxidase on hypoxanthine [10]. The reaction mixture contained 10 mM Tris-HCl, pH 7.4, 100 μM hypoxanthine, 10 μM EDTA, and 4 mU of xanthine oxidase in a final volume of 0.5 ml. To monitor the generation of O_2^-, 100 μM cytochrome C was added to this reaction mixture and its reduction was monitored spectrophotometrically at 550 nm [10]. To generate OH ·, 100 μM each of $FeCl_3$ and EDTA were added to the O_2^- generating system [10, 13]. The OH · generated under these conditions was measured after chemically trapping the radical with 2 mM salicylic acid and analyzing the hydroxylated products, 2,3-and 2,5-dihydroxybenzoic acids using a high pressure liquid chromatograph (HPLC) (Waters) equipped with an Altex ulrasphere 3 μm ODS column and an electrochemical detector as described previously [10].

Exposure of FABP to O_2^- and OH ·
Two nmols of purified and delipidated FABP was incubated either with the O_2^- or with the OH · radical generating system (0.5 ml) at 37° C for 30 minutes. To measure the binding of oleate, the generation of free radicals was quenched by the additions of SOD and catalase (50 units each). For SDS-PAGE experiments, 50 μl of 20% SDS was added to terminate the reaction. For 7.5% PAGE under non-denaturing conditions, incubations were terminated by rapid cooling of the reaction mixture in a dry ince-acetone bath. Control experiments were performed simultaneously by omitting hypoxanthine in the reaction mixture.

Measurement of oleate binding by FABP
The reaction mixture contained increasing amounts of [1-^{14}C] oleic acid Na^+ salt, 10,000 cpm, 2 nmol FABP (control or exposed to O_2^- or OH ·), 1.5 mM dithiothreitol and 100 mM Tris-HCl, pH 7.4, in a total volume of 0.5 ml [12]. Binding was allowed to occur for 5 minutes at 37° C, after which the reaction tubes were cooled in an ice bath. The unbound oleate was removed by mixing with 200 μl of ice-cold Lipidex 1000 in buffer (1 : 1 suspension) for 10 minutes at 4° C [11]. The radioactivity remaining in the supernatant was determined and pmol oleate bound/μg FABP was calculated by Scatchard analysis after correcting for non-specific binding.

SDS-PAGE and non-denaturing PAGE
The free radical reaction was terminated either by the additon of SDS or by rapid cooling as previously discussed. The reaction mixtures were lyophilized, and 100 μl sample buffer (0.675 M Tris, 2% SDS, 20% glycerol, 10% β-mercapto-ethanol, pH 6.9) for SDS-PAGE or (0.15 M Tris, with 35% sucrose w/v) for non-denaturing PAGE was added. FABP was then subjected to either 15% SDS-

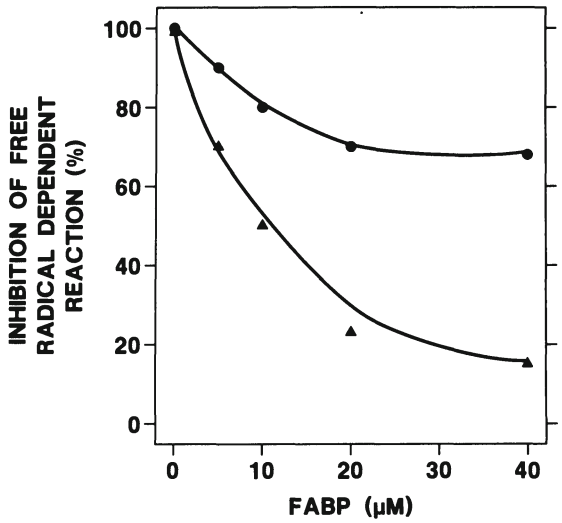

Fig. 1. Effect of varying concentrations of FABP on the free radical dependent reactions. Indicated amounts of FABP was included either in the O_2^- (●——●) or OH · generating system (▲——▲) and the cytchrome C reduction by O_2^- and 2,3, dihydroxybenzoic acid formation from OH · were measured as described in the text.

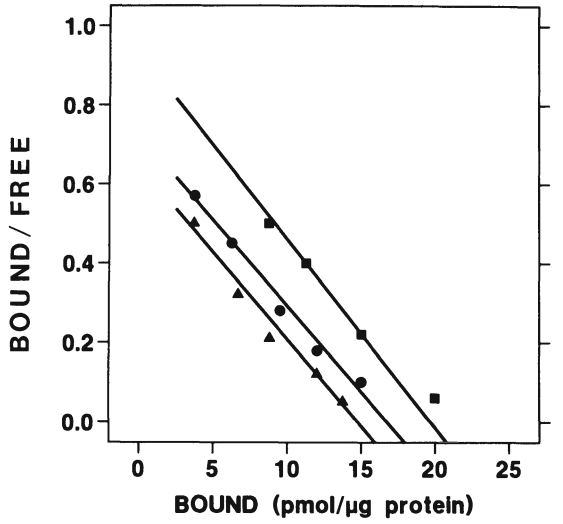

Fig. 2. Scatchard analysis of the binding of oleate by the FABP exposed to oxygen radicals. 2 nmol of FABP was exposed to either O_2^- (●——●) of OH · (▲——▲) for 30 min. following which they were incubated with 0.1 to 3.0 μM [1-^{14}C] oleate. After equilibration, protein – bound and unbound oleate were separated by using Lipidex at 0° as described under text. For the control experiments hypoxanthine was omitted (■——■).

PAGE as described by Laemmli [14], or to 7.5% non-denaturing PAGE in an glycine-chloride system [15]. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 and destained as described previously [14].

Results

The O_2^- generated by the action of xanthine oxidase on hypoxanthine was examined by the reduction of cytochrome C. In our assay conditions as much as 2.5 nmol O_2^-/min was generated. The generation of OH · was monitored by trapping it with salicylic acid and analyzing the hydroxylated products 2,3- and 2,5-dihydroxybenzoic acid using HPLC [10]. Using a standard curve of an equimolar mixture of 2,3- and 2,5- dihydroxybenzoic acid, the amount of hydroxylated products of salicylic acid were calculated to be about 500 pmol/10 min of reaction (data not shown). These results suggest that at least 500 pmol of OH · was generated under the present assay conditions. Before examining whether the exposure of FABP to either O_2^- or OH · affects its binding of fatty acids, the O_2^- dependant reduction of cytochrome C and OH · dependent formation of dihydroxybenzoic acids were examined in the presence of increasing amounts of FABP. The results displayed in Fig. 1 show that FABP reduced the free radical targeted reactions. A maximum inhibition of O_2^- dependent reduction of cytochrome C was observed at 40 μM FABP concentration. The same amount of FABP inhibited 85% of OH · dependent dihydroxybenzoic acid formations. These results suggest that FABP can be attacked by either O_2^- or OH ·.

Chemically generated O_2^- or OH · has been demonstrated to change the primary, secondary and tertiary structure of several proteins, including bovine serum albumin, an extracallular fatty acid-binding protein [16, 17]. Since such structural changes in a protein could render it non-functional, the effect of O_2^- or OH · on the fatty acid binding ability of FABP was examined. The binding of oleate by the O_2^- or OH · treated FABP as a function of the total oleate concentration was measured and the Scatchard analysis of the binding isotherms are shown in Fig. 2. The exposure of FABP to O_2^- and OH · decreased the oleate binding capacity

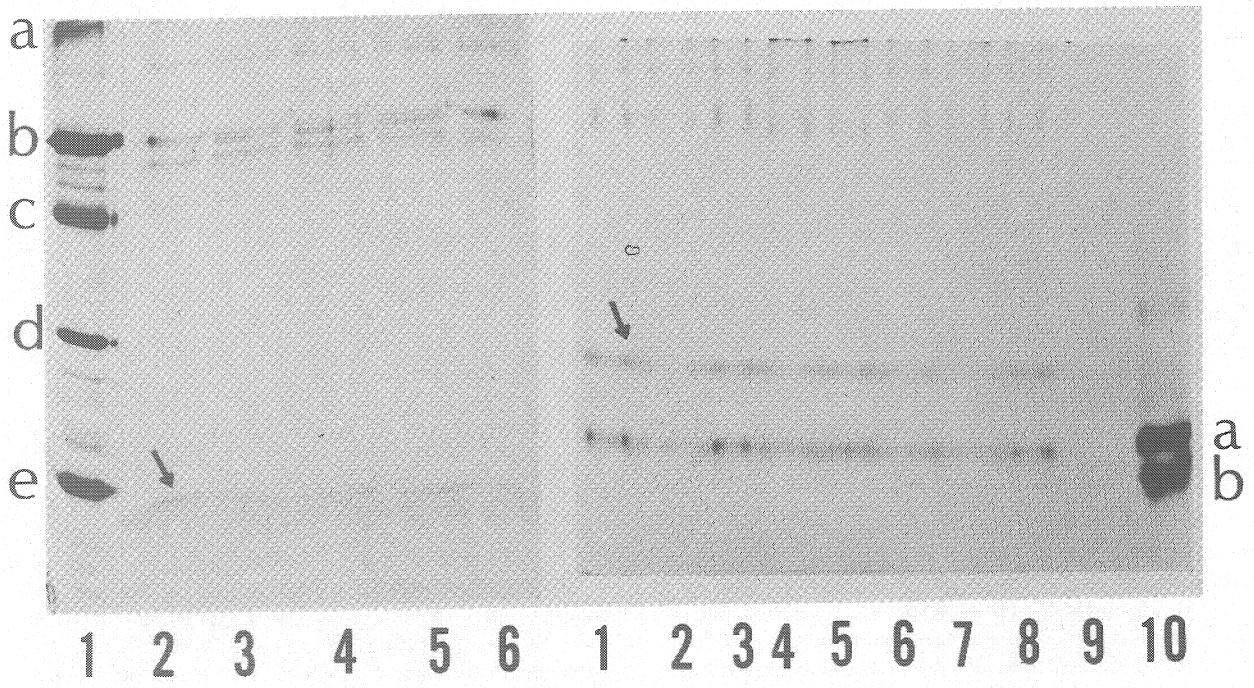

Fig. 3. Polyacrylamide gel electrophoresis of FABP exposed to oxygen free radicals. *Left.* 2 nmol FABP was exposed to either O_2^- or OH · and subjected to SDS-PAGE as described under text, from left to right, Lane 1: protein standards; BSA (67,000 K.D.), ovalbumin (45,000 K.D.), chymotrypsin (25,000 K.D.), RNase (13,700 K.D.). Lane 2: FABP + XO (hypoxanthine omitted), 3 + 4 FABP exposed to O_2^-, 5 + 6 FABP exposed to OH · *Right* 2 nmol FABP was exposed to either O_2^- or OH · and subjected to non-denaturing PAGE as described under text from left to right, Lanes 1,2 and 3: FABP exposed to OH ·, Lanes 4, 5 and 6: FABP exposed to O_2^-, Lanes 7, and 8: FABP + XO (hypoxanthine omitted), Lane 9: Blank, Lane 10: protein standards a) myoglobin, b) cytochrome C.

from 21.5 pmol/μg protein to 18.0 and 15.5 pmol/μg protein, respectively.

The polyacrylamide gel electrophoresis of the free radical exposed FABP was carried out under denaturing and non-denaturing conditions to examine whether O_2^- or OH · caused any degradation or aggregation of FABP. The SDS – gel electrophoresis pattern included in Fig. 3 shows that the staining intensity of FABP (14,000 D) was not decreased to any appreciable extent by exposure to either O_2^- or OH ·. These results suggest that these free radicals did not cause any degradation of FABP.

The non-denaturing gel pattern of free radical exposed FABP shows that there is neither a decrease in the intensity of the two FABP bands nor an appearance of a new protein band with a mobility either greater or less than FABP (Fig. 3). These results suggest that, at least under the present assay conditions, O_2^- or OH · did not cause aggregation or change in the net ionic charge of FABP.

Discussion

Fatty acid-binding proteins identified in the cytosol of several tissues including heart have a high affinity for fatty acids and their esters [3]. Several studies carried out to examine the biological role of FABP suggest its function 1) as an intracellular transport protein of fatty acids between subcellular organelles [4], 2) as an intracellular protein which reverses the acyl-CoA mediated inhibition of membrane bound and cytosolic enzymes [17] and 3) as an acceptor protein of fatty acyl-CoA which serves as a better substrate to glycerophosphate acyl transferase during phospholipid biosyntheses [5].

The generation O_2^- derived free radicals during

the reperfusion of ischemic-myocardium [6] and the ability of O_2^- and OH · to degrade and aggregate proteins like BSA, cytochrome C, and myoglobin [15, 16] raises following questions: 1) Is FABP also susceptible to the attack by O_2^- or OH ·? 2) Does the exposure of FABP to O_2^- or OH · at near physiological concentrations cause either the degradation or the aggregation of FABP molecules? The present study attempts to answer these questions.

Recently, Davies *et al.* [15, 16] observed a significant loss of cysteine, histidine, tryptophan, phenylalanine as well as degradation and aggregation of bovine serum albumin by exposing 1 nmol of bovine serum albumin to 10 nmol of OH ·. However, a ratio of 10 nmol free radical to 1 nmol protein appears to be non-physiologic since mitochondrial or sub-mitochondrial particles produce about 3–5 nmol of O_2^-/min/mg or per 20 nmol (based on an average molecular weight of 50,000) protein. According to the Fenton reaction given below, 1 nmol of O_2^- can generate 1 nmol of OH · maximally.

$$O_2^- + O_2^- + 4H^+ \rightarrow 2H_2O_2$$
$$2H_2O_2 + 2Fe^{3+} \rightarrow 2OH\cdot + 2OH^-$$

Thus, in the myocardial cell 1 nmol of protein cannot be attacked by more than 0.15 to 0.25 nmol of OH ·. Therefore, exposing of about 2 nmol FABP with 500 pmol of OH · in the present study are within physiological concentrations.

The results in this paper show that FABP was able to inhibit the O_2^- dependent cytochrome C reduction and OH · radical dependent dihydroxybenzoic acid formations. The greater ability of FABP to block OH · dependent reaction as compared to O_2^- is consistent with the observation of Davies *et al.* [15, 16], who also showed that OH · is a more reactive free radical than O_2^- in causing the protein damage. The ability of FABP to inhibit OH · dependent reactions suggests that it may protect the membrane phospholipids from the free radical attack and thus can act as a biological free radical scavenger. However, such a role for a FABP can only be of biological significance if the free radical attack does not result in a loss of fatty acid binding capacity. The results presented in this paper indicate that the oleate binding capacity was decreased by only 27% and14% by OH · and O_2^-, respectively, when 2 nmol of FABP was exposed to 0.5 nmol OH ·. Therefore, if 20 nmol of FABP is exposed to 0.5 nmol of OH · the decrease of oleate binding capacity should be less than 27% as observed in the present study. However, 40 μM (20 nmol) FABP scavenged about 85% of OH ·. Thus, based on the present results, it is tempting to suggest that myocardial FABP can act as a free radical scavenger and thus may protect the integrity and function of biological membranes.

Glatz and his coworkers [19] recently demonstrated that either Ca^{2+} paradox or reperfusion of ischemic myocardium released FABP from myocardium. However, the release of FABP during reperfusion of ischemic myocardium was only 2.9% of total cytosolic FABP content, as compared to 56% in Ca^{2+} paradox Thus, their results suggest the loss of FABP during reperfusion may not play a significant role in the reperfusion injury. On the other hand, the results of the present study indicate that fatty acid binding abilities of FABP is not markedly lowered by free radical attack. Thus, the ability of FABP to act as a free radical scavenger may be beneficial to the reperfused myocardium.

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Fatty acid-binding protein expression in the liver: its regulation and relationship to the zonation of fatty acid metabolism

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Key words: dietary fat, fatty acid-binding protein, fatty acid metabolism, isolated liver cells, liver acinus, metabolic zonation

Summary

Liver fatty acid-binding protein (L-FABP) is expressed in a declining gradient between the portal and central zones of the liver acinus. This paper discusses the results of experimental studies which address the questions: (a) What factors regulate L-FABP expression in liver and produce its acinar gradient? (b) What is the relationship between the acinar gradient of L-FABP and acinar gradients in the transport and metabolism of long-chain fatty acids? Both high-fat diets and clofibrate-treatment increase L-FABP proportionally at both extremes of the liver acinus and the small intestine, with preservation of the L-FABP gradient in both tissues. Female rats differ from males, however, in showing a greater hepatic abundance of L-FABP which is expressed almost equally throughout the acinus. Dietary studies show that L-FABP is induced with increased fatty acid flux derived from dietary fat but not from *de novo* hepatic fatty acid synthesis. Studies of the synthesis and utilization of fatty acids by hepatocytes isolated from the periportal and pericentral zones of the liver acinus suggest that the acinar gradient of L-FABP is not associated with differences in the instrinsic capacity of zone 1 and zone 3 hepatocytes to utilize or synthesize fatty acids. In addition, studies of the acinar uptake pattern of a fluorescent fatty acid derivative by isolated perfused livers indicate that the acinar distribution of L-FABP does not determine the pattern of fatty acid uptake in the intact acinus. Rather, the acinar gradient of L-FABP is most likely to represent a response to physiological conditions existing in the intact acinus which may include gradients in the flux of fatty acids, fatty acid metabolites and hormones.

Abbreviations: ALT – Alanine Aminotransferase, FABP – Fatty Acid Binding Protein, I-FABP – Intestinal-type Fatty Acid Binding Protein, L-FABP – Liver-type Fatty Acid Binding Protein, 12-NBD-stearate – 12-(N-methyl)-N-(7-nitrobenzo-2-oxa-1, 3,-diazol-4-yl)amino)-octadecanoic acid

Introduction

The abundant cytosolic fatty acid binding proteins (FABP) present in the liver and intestine display striking regional differences in their expression. Both liver-type FABP (L-FABP), and the intestine-specific FABP (I-FABP) are expressed in declining gradients from the jejunum to the ileum and also decline in abundance from the villus tips to the crypt cells of the intestinal villi [1–8]. In the liver, L-FABP is expressed in a declining gradient from the periportal (acinar zone 1) hepatocytes to the pericentral (acinar zone 3) hepatocytes in rats [9, 10] mice [5] and humans [11].

The existence of gradients in the expression of FABP in liver and intestine affords a means to experimentally address questions relating to the factors responsible for the regulation of these pro-

teins as well as to the relationship of FABP expression to regional differences in the transport and utilization of fatty acids. Recent elegant studies from Gordon's laboratory on the expression in transgenic mice of the transcribed portion of the human growth hormone gene linked to portions of the 5′ non-transcribed region of the rat FABP genes have begun to identify the cis-acting DNA sequences responsible for regional differences in FABP expression [5–7]. This work has shown that, in the case of L-FABP, nucleotides − 596 to + 21 of the L-FABP gene ('short promoter') direct the expression of the growth hormone reporter gene to hepatocytes as well as epithelial cells in the small intestine, colon and kidney in transgenic mice [5]. Aberrant expression of the reporter gene in the colon and kidney is suppressed by elements present in nucleotides − 4000 to − 597 of the L-FABP gene. However, sequences present within the short promoter alone are sufficient to produce patterns of reporter gene expression that mimic the normal jejunal-to-ileal small intestinal gradient and portal-to-central hepatic gradients in transgenic mice [5]. Specific trans-acting factors that participate in regulating the gradient expression of L-FABP are yet to be identified. Such factors might include circulating hormones as well as fatty acids, the latter entering hepatocytes from the circulation or, in the case of the small intestinal epithelium, from both the intestinal lumen and the circulation.

The architecture of the liver is viewed either as comprising hexagonal units (lobules) bounded at their vertices by the portal tracts, or as compromising microcirculatory units or acini, surrounding each terminal portal venule [12]. The liver acinus is conventionally divided into 3 zones: zone 1, corresponding to the periportal region, zone 3 corresponding to the pericentral region surrounding the terminal hepatic venule and zone 2, being intermediate between zones 1 and 3. There is considerable evidence for zonal heterogeneity in numerous enzymes and metabolic functions in the liver lobule [12–14]. Zonal differences in certain enzymes of lipid metabolism have also been described [15, 16]. In view of the board role proposed for L-FABP in the transport and utilization of fatty acids [17], it is possible that the declining zone 1 to zone 3 acinar gradient of L-FABP reflects a functional relationship of this protein to one or several pathways of fatty acid metabolism which are also heterogeneously distributed throughout liver acinus. Furthermore, the acinar gradient of L-FABP may, hypothetically speaking, reflect a response to fluxes of metabolites that differ between the portal and central zones in a manner determined by the unequal zonal activities of particular metabolic pathways of fatty acid metabolism. This paper describes studies which have examined the influence of dietary, pharmacological and hormonal factors on the acinar expression of L-FABP, and which have investigated the relationship between the acinar gradient of L-FABP and the uptake and metabolism of fatty acids in hepatocytes from the portal and central zones of the liver acinus.

Regulation of L-FABP in the liver and intestine

Table 1 provides a summary of the factors which increase the abundance of L-FABP in the liver and intestine. In all instances comprehensively studied to date, an increase in L-FABP abundance has

Table 1. Factors associated with L-FABP modulation in liver and intestine[a]

	Protein		Turnover		mRNA	
	liver	intestine	liver	intestine	liver	intestine
Peroxisome proliferators	↑	↑	no change	no change	↑	↑
High-fat feeding	↑	↑	?	?	?	?
Female sex steroids	↑	no change	no change	↑	↑	↑

[a] Adapted from data in refs. 2, 17, and 18.

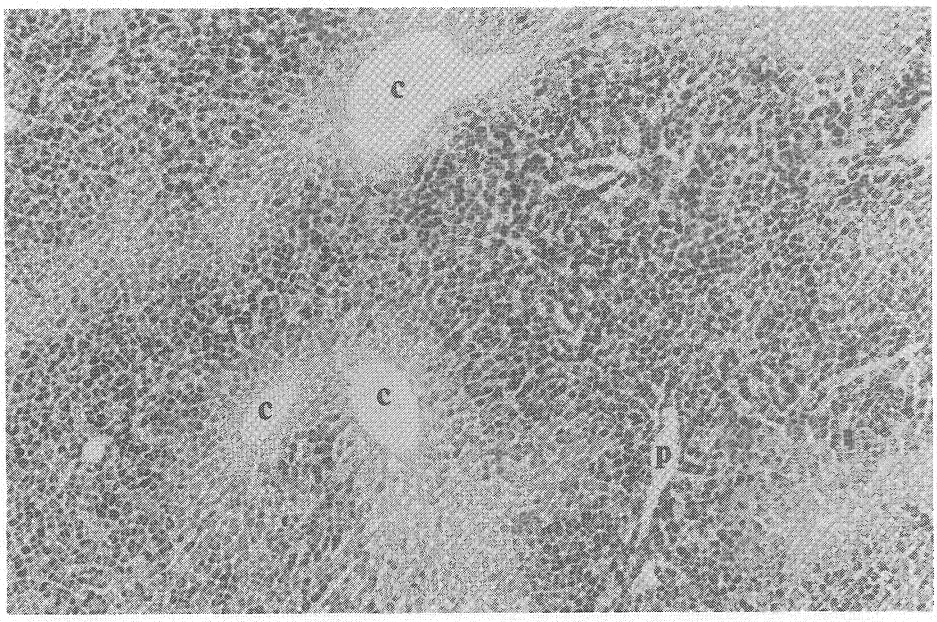

Fig. 1. Immunohistochemical staining of L-FABP in the male rat liver acinus demonstrating the portal (zone 1)-to-central-(zone 3) acinar gradient in L-FABP abundance. L-FABP was stained using a specific rabbit anti-rat L-FABP antiserum by the immunoperoxidase method. Central venules (c) and portal venules (p) are indicated.

been secondary to increased synthesis of the protein accompanied by an increase in its mRNA [2]. Curiously, in females, L-FABP abundance in the intestine is similar to that in males, but both the turnover of L-FABP and its mRNA are increased in female intestine.

Diets rich in fat or the administration of the peroxisome proliferator hypolipidemic drug, clofibrate, increase the hepatic and intestinal abundance of L-FABP [2, 17, 18]. In the case of the intestine, both the jejunal and ileal content of this protein are increased, and the proximal-to-distal gradient is maintained. The relationship between the effect of high-fat diets and clofibrate is extremely interesting, as peroxisome proliferators may exert their biological effects via the formation of CoA esters [19] and through the pertubation of cellular fatty acid metabolism to produce a state of fatty acyl 'substrate overload' [20]. Indeed, the effects of peroxisome proliferators in many ways appear to be an exaggerated version of the adaptive response of liver cells to diets rich in very long-chain fatty acids [21].

Recent studies in the author's laboratory have further examined the regulation of L-FABP by dietary factors (Bass NM, Manning JA, unpublished observations). When rats were fed a low-fat, high-sucrose diet, an expected marked increase in hepatic lipogenesis occurred. This lipogenic diet led to no change in L-FABP abundance in the livers of male rats. L-FABP in the ileum also showed no change, but levels of the protein fell to 60% of chow-fed control values in the jejunum. This is similar to the response of I-FABP to a low-fat diet [1]. When female animals were fed the same diet, hepatic L-FABP levels, normally ~ 1.4-times higher in females than in males, declined to the same level seen in males. Several conclusions may be tentatively drawn from these findings. First, although L-FABP abundance is increased by dietary fat, this protein is not increased, and in some instances is even decreased, by a lipogenic diet. It may be important in this regard that common to both clofibrate-treatment and high-fat feeding, is an increase in hepatic β-oxidation of fatty acids [22, 23]. Conversely, this pathway for fatty acid disposal is inhibited in animals fed a lipogenic diet [24]. The gradient for L-FABP is reduced in the small

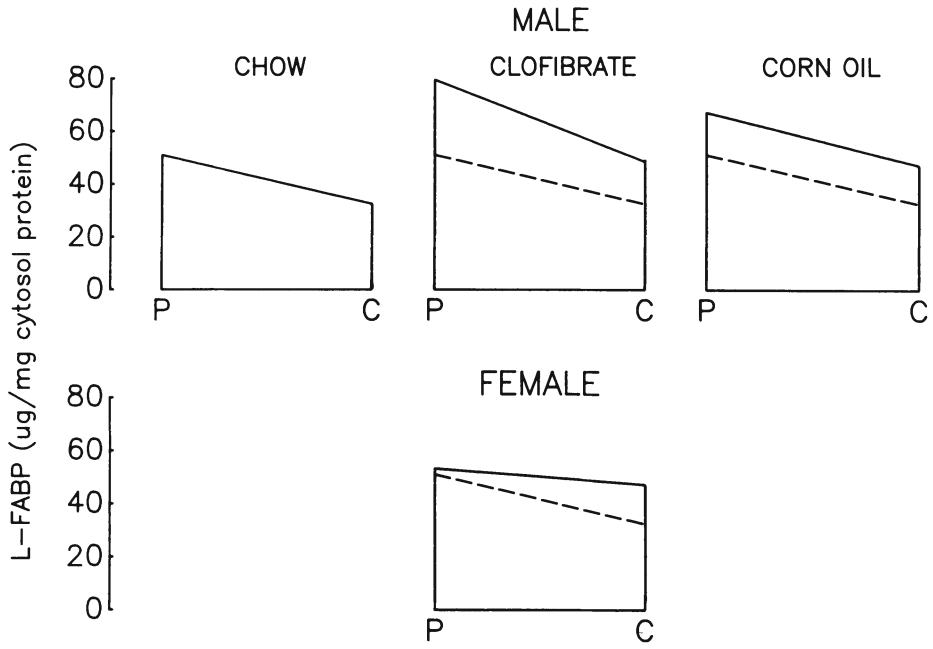

Fig. 2. Gradients of L-FABP in the rat liver acinus. L-FABP was measured in perfusate effluents from livers pulse-perfused with digitonin in either anterograde or retrograde directions in order to permeabilize zone 1 or zone 3 hepatocytes, respectively. Mean values for L-FABP in effluents from permeabilized periportal (zone 1) cells (P), or pericentral (zone 3) cells (C), are shown for male rats fed stock chow, treated with clofibrate or fed a high-fat (30% corn oil, w/w) diet, and for female rats fed a stock chow diet. The dashed line represents the gradient in chow-fed males for ease of comparison. Data are adapted from refs. 10 and 25.

intestine by a low-fat lipogenic diet, as a result of a fall in L-FABP abundance in the jejunum without a change in ileal L-FABP. This suggests that L-FABP is modestly induced in the jejunum under conditions of normal dietary fat intake (~ 5–6% fat, w/w) while its ileal expression is apparently constitutive under these conditions. The level of L-FABP expression in male liver appears to be similarly constitutive with respect to dietary fat. On the other hand, the fall in L-FABP abundance in the livers of females fed a lipogenic diet may point to a role for plasma fatty acid flux in the production of the sex difference in hepatic L-FABP expression.

Gradients of L-FABP in the liver acinus

The intestine readily lends itself to the quantitation of differences, in and pertubations to, the expression of L-FABP along its horizontal axis. The investigation of gradients along the sinusoidal axis of the liver lobule is obviously more difficult, and these have largely been studied by immunohisto-

Table 2. Heterogeneity of lipid metabolism in the liver acinus[a]

Predominantly Periportal/Zone 1	Predominantly Pericentral/Zone 3
Mitochondrial β-oxidation	Peroxisomal β-oxidation
Cholesterol synthesis	Ketogenesis
L-FABP	Fatty acid synthesis (lipogenesis)

[a] Adapted from refs. 15 and 16.

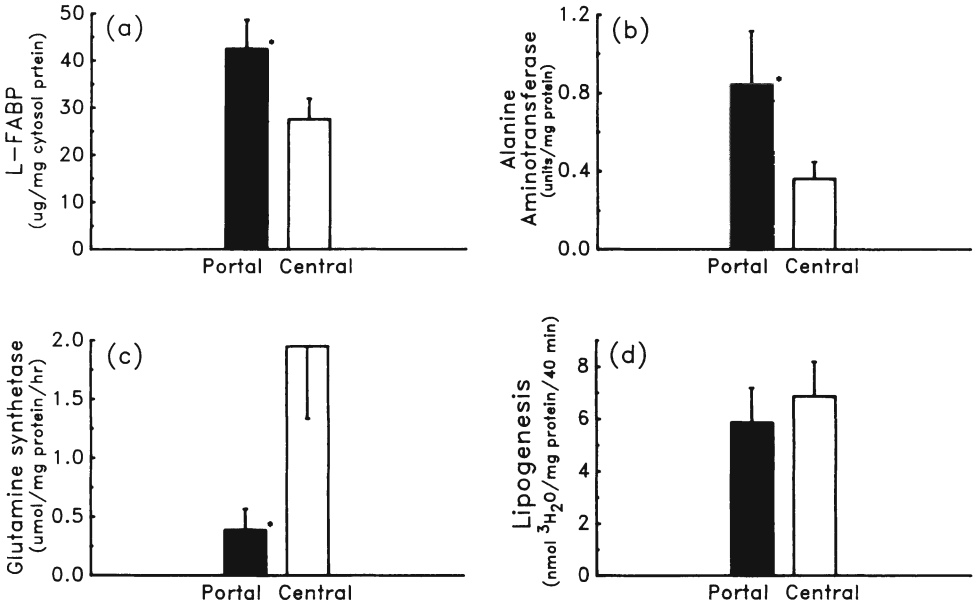

Fig. 3. L-FABP abundance, marker enzyme activities and lipogenesis in isolated acinar zone 1 and zone 3 hepatocytes. Zonally enriched hepatocytes were isolated by digitonin/collagen perfusion as described in the text. (a) L-FABP, (b) alanine aminotransferase, (c) glutamine synthethase and (d) lipogenesis were determined as described in the text. Values represent means ± S.D. for separate determinations made with periportal (zone 1) cells (closed bars), and pericentral (zone 3) cells (open bars) obtained from 8–12 animals. * Periportal vs pericentral hepatocytes: $p < 0.001$. Data are from ref. 25 and Bass NM, Manning JA, unpublished data.

chemical techniques. Immunohistochemical studies have adequately demonstrated the existence of a portal-(zone 1)-to-central-(zone 3) gradient in L-FABP, as well as marked heterogenity in the intensity of L-FABP immunostaining in hepatocytes within a given acinar zone [5, 9–11, Fig. 1]. In the human fetus, L-FABP immunoreactivity is uniformly distributed throughout the acinus [11]. The acinar gradient of L-FABP is therefore likely to develop in response to nutritional and/or hormonal influences that arise following birth. Of considerable interest, L-FABP immunostaining appears to be particularly strong in hepatocytes undergoing mitotic division [5, 9] leading to some speculation regarding a role for this protein in the regulation of the cell growth cycle. L-FABP expression in the liver is also confined to hepatocytes and is not observed in bile ductular epithelium, sinusoidal endothelial cells, Kupffer cells or fat-storing Ito cells [5, 10, 11]. The absence of L-FABP in the latter has recently been confirmed in our laboratory in isolated, pure populations of Ito cells in culture (Kaikaus RM, Friedman S, Bass NM, unpublished observations). For the purpose of obtaining a more quantitative estimate of the dimensions of the L-FABP acinar gradient and to determine the changes in this gradient under the influence of various regulatory factors, we adopted the approach of selective zonal permeabilization with digitonin developed by Quistorff *et al.* [25]. In these studies [9], L-FABP as well as the zone 1 hepatocyte marker enzyme, alanine aminotransferase (ALT), were measured in the effluent from livers perfused for brief periods in either anterograde (via the portal vein) or retrograde (via the hepatic veins) directions with digitonin. Livers perfused in this manner release the cellular contents of zone 1 (periportal) cells during anterograde perfusion and of zone 3 (pericentral) cells during retrograde perfusion. The results of this approach to quantitating the L-FABP acinar gradient and the effects of clofibrate high-fat feeding and female sex on the gradient are shown in Fig. 2. The ratio of L-FABP between portal and central hepatocytes in untreat-

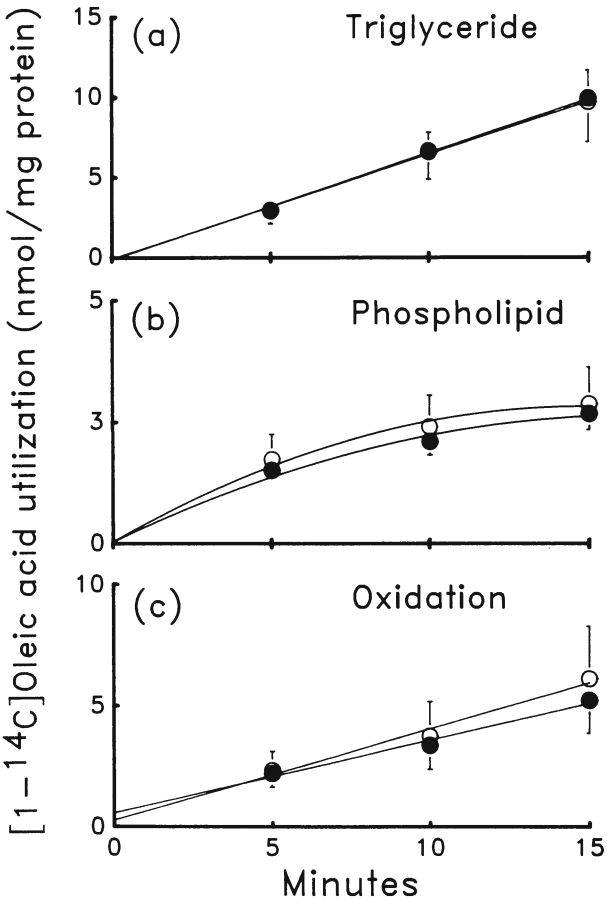

Fig. 4. Oleic acid utilization by isolated acinar zone 1 and zone 3 hepatocytes. Zonally enriched hepatocytes were isolated as described in the text. Incorporation of [1-^{14}C] oleic acid (0.3 mM), into (a) triglyceride, (b) phospholipids and (c) water-soluble oxidation products was measured at the times indicated as described in the text. Values represent means ± S.D. for separate determinations made with (●) periportal (zone 1), or (○) pericentral (zone 3) hepatocytes obtained from 6 to 7 animals. Data are from ref. 25, and Bass NM, Manning JA, unpublished data.

ed male rats is 1.6 : 1 which is rather less dramatic than might have been predicted from the visual appearance of this gradient in immunohistochemical sections. On the other hand, the portal-to-central ratio in ALT activity obtained in the same animals was 6 : 1. This confirms the selectively of the zonal permeabilization obtained with the digitonin technique. The effects of clofibrate and fat-feeding on the acinar gradient of L-FABP in male rats were qualitatively quite similar to their effects on on the gradient of L-FABP in the intestine [10, 17, 26], in that L-FABP was increased in a proportional manner at both extremes of the acinus with preservation of the acinar gradient. Unfortunately, similar studies in animals fed a lipogenic diet were unsuccessful due to technical difficulty in obtaining uniform perfusion of the livers which were considerably infiltrated with fat. In female rats, an acinar gradient was evident on immunohistochemistry, but was far less pronounced than in male animals [10]. The attenuation of the gradient in females was confirmed by digitonin perfusion studies (see Fig. 2). Thus, in females, although L-FABP abundance in zone 1 cells was found to be similar to that in males, the pericentral (zone 3) abundance was increased above the corresponding male levels in this region of the acinus. Therefore, the greater abundance of L-FABP in female vs male liver reflects a distal acinar increment in expression of the protein. The hepatic expression of L-FABP thus shows sex differences that are both quantitative and qualitative.

Gradients in the expression of enzymes of fatty acid metabolism in the liver acinus

The existence of zonal compartmentation (zonation) of carbohydrate metabolism in the hepatic acinus has been wel documented [13]. Evidence for similar zonation of lipid metabolism has also been provided by a number of studies [15, 16], and has been based, in the main, on immunohistochemical and microchemical determination of the zonal distribution of specific enzymes within the acinus. Table II summarizes current concepts regarding the zonation of lipid metabolism within the hepatic acinus. Several points regarding these concepts are worth emphasizing. Firstly, it is worth noting that the acinar gradient of L-FABP corresponds in direction to the proposed gradient of mitochondrial β-oxidation activity. The acinar distribution of L-FABP also coincides with the periportal predominance of key enzymes in cholesterol biosynthesis. A role for L-FABP in cholesterol biosynthesis has been suggested by some [27, 28] but not all [29] studies. Secondly, peroxisomes are normally more abundant in the pericentral hepatocytes [16], and a

marked central-to-portal gradient in peroxisome number is obtained in animals treated with peroxisome proliferators [30]. It appears, therefore, that these agents produce hepatic acinar gradients for peroxisomes and L-FABP that are essentially inclined in opposite directions. A simple interpretation of the significance of these opposing patterns of induced gradients for L-FABP and peroxisomes cannot be offered at present. They may, for example, indicate a dissociation between peroxisomal utilization of fatty acids and the function of L-FABP, or conversely, a 'complementary' regulation of L-FABP and peroxisomes, underlying a functional relationship between the protein and these organelles. Finally, it must be noted that the orientation of the acinar gradients for L-FABP is opposite to the acinar gradient proposed for lipogenesis (Table 2) based upon the histochemical patterns of enzymes participating in fatty acid synthesis as well as the enzymes providing reducing equivalents for lipogenesis. This may correlate with the lack of effect, or negative effect, of lipogenic diets on L-FABP abundance discussed earlier, but offers little insight into a functional role [31] or lack thereof, for L-FABP in fatty acid synthesis. Furthermore, the zonal pattern of lipogenic activity remains controversial. A recent study by Evans and colleagues [32] using the digitonin permeabilization technique found, in distinct contrast to histochemically defined patterns [15], a clear-cut zone 1 predominance in the mass and activity of the principal enzymes of fatty acid synthesis (acetyl-CoA carboxylase, fatty acid synthase and ATP-citrate lyase) in male rat liver. The livers of female rats, however, displayed similar high periportal and pericentral contents of these enzymes. Thus, the data from Evans *et al.* [32] indicate the existence of a sex difference in the zonation of lipogenesis which is remarkably congruent with the sex differences in the acinar expression of L-FABP (see Fig. 2).

The relationship between L-FABP abundance and rates of fatty acid utilization and synthesis in isolated acinar zone 1 and zone 3 hepatocytes

In order to further clarify the relationship between the gradient of L-FABP and the activities of major pathways of fatty acid metabolism within the acinus, studies were performed in the author's laboratory using hepatocytes isolated predominantly from acinar zones 1 and 3 [26]. Isolation of zonally-enriched populations of hepatocytes was accomplished using more extended anterograde or retrograde liver perfusion with digitonin to selectively destroy cells in zone 1 (plus zone 2) or in zone 3 (plus zone 2). The viable cells remaining in the selectively spared zone, downstream from the direction of perfusion, were then isolated by collagenase perfusion [33]. Zonal hepatocyte populations isolated by this method were $>85\%$ viable on the basis of trypan blue exclusion. Alanine aminotransferase [25, 33] and glumatine synthetase [34] activity were used as periportal and pericentral marker enzymes respectively, and L-FABP was measured in the soluble protein fraction by immunoassay. Rates of lipogenesis were measured from the incorporation of ^3H_2O into saponifiable lipids [35]. Rates of fatty acid utilization was measured by incubating cells with [1-^{14}C] oleic acid (0.3 mM complexed with 0.15 mM bovine serum albumin) and determining rates of incorporation into various lipid classes, separated by thin-layer chromatography, and water-soluble (oxidation) products [36].

As shown in Fig. 3, L-FABP and ALT activity were significantly greater in periportal (zone 1) hepatocytes compared with pericentral (zone 3) hepatocytes. Conversely, glumatine synthetase activity was significantly greater in the pericentral cells. Thus, on the basis of L-FABP abundance, and the differential distribution of established zonal marker enzymes, zonally-enriched populations of isolated hepatocytes were obtained using the digitonin-collagenase perfusion technique. Rates of fatty acid synthesis, however, were similar in the two populations of hepatocytes (Fig. 3). Furthermore, as shown in Fig. 4, rates of [1-^{14}C] oleic acid incorporation into tryglycerides, phospholipids

and oxidation products were virtually identical for zone 1 and zone 3 hepatocytes. When lower concentrations of oleic acid were used, there still remained no observable difference in the rates of fatty acid utilization between zone 1 and zone 3 isolated hepatocytes.

From these studies, it can be concluded that rates of lipogenesis and fatty acid utilization fail to correlate with L-FABP abundance in isolated zone 1 and zone 3 cells, at least in a manner that is manifestly dependent on L-FABP concentration. Thus, at its extant abundance within the hepatic acinus, L-FABP does not appear to be rate limiting to these pathways of fatty acid metabolism. There are both caveats and conclusions that bear upon these findings. Firstly, the absence of 'gradients' in the metabolic activities measured in these isolated cell studies does not preclude the existence of such gradients in the intact acinus. Gradients of hormones, PO_2 and substrates within the intact, sinusoidally perfused acinus may be of critical importance to the development and maintenance of gradients *in vivo*. Thus, although we were unable to find intrinsic differences in the rates of oleic acid conversion to oxidation products in isolated zone 1 and zone 3 hepatocytes, this does not preclude the existence of a zone 1 predominance for fatty acid β-oxidation activity in the intact acinus [14]. It has also been amply shown that the zonal pattern of certain metabolic activities within the acinus may be rapidly reversed following reversal of the direction of perfusate flow in the liver [13, 14]. Therefore, the zonal predominance of certain enzymes and proteins, including L-FABP, may reflect a primarily adaptive rather than a deterministic phenomenon.

The relationship between the acinar gradient of L-FABP and the acinar pattern of fatty acid uptake

The foregoing discussion returns us to the question of which regulatory factors might be responsible for the evolution and maintenance of the L-FABP acinar gradient. As pointed out earlier, both fatty acid flux from the plasma and factors derived from, or related to, fatty acid flux through the β-oxidation pathway are attractive, but clearly not exclusive, candidates for such a role.

Reported hepatic extraction fractions for albumin-bound long-chain fatty acids are of the order of 30–40% in the fed state [37, 38] and 50% during fasting [37]. This indicates that for fatty acid flux from plasma, a zone 1-to-zone-3 ratio ranging from 1–4 : 1 to 2 : 1 may normally prevail in the liver. This acinar gradient of fatty acid flux is very similar in magnitude to the L-FABP acinar gradient. In order to better define the relationship between the acinar pattern of L-FABP expression and the uptake of fatty acids, studies were conducted with the fluorescent fatty acid derivative, 12-NBD-stearate using isolated liver perfusion and fluorescent microscopy [39]. 12-NBD-stearate binds strongly to pure L-FABP, and associates selectively with this protein in hepatic cytosol on gel filtration chromatography [39]. When perfused complexed to albumin via the portal vein, 12-NBD-stearate fluorescence was seen to decline from the portal to the central zones. When the direction of perfusion was reversed, however, greater fluorescence was observed in pericentral zone. These findings demonstrate that the acinar expression of L-FABP is more likely to represent a response to, than a determinant of, the acinar pattern of fatty acid influx existing under normal conditions of sinusoidal perfusion. This implies a situation similar to that in the intestine, where the gradients of L-FABP along the length of the intestine and the villi correspond to regional differences in exposure to, and inward flux of fatty acids assimilated from the diet [40].

Summary and conclusions

Although many questions still remain to be answered regarding the regulation of L-FABP, including factors which are responsible for the gradients in its expression, some preliminary conclusions may be drawn from the experimental work discussed above. Firstly, L-FABP shows both constitutive and inducible types of expression in the liver and intestine. There are also both quantitative and qualitative sex-differences in the hepatic expression of L-FABP, the basis for which is still unclear.

As regards the role of fatty acids in the regulation of this protein, fatty acids derived from the diet but not from the novo hepatic synthesis lead to an increase in L-FABP abundance. Furthermore, a relationship is evident between L-FABP induction and states of increased fatty acid oxidation which remains to be fully explored. Finally, a relationship has not emerged between the acinar gradient of L-FABP and the activity of pathways of fatty acid metabolism in isolated zonal hepatocytes. Although such a relationship may exist in the intact acinus in vivo, it is likely that this will reflect mutual adaptation of the expression of L-FABP and pathways of fatty acid metabolism to conditions that exist in the intact acinus. These may include acinar gradients in the hepatocellular influx of fatty acids, differences along the acinus in the hepatocellular flux of fatty acid metabolites, or acinar gradients in the extraction of hormones which modulate pathways of fatty acid metabolism.

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Effects of linoleic and gamma-linolenic acids (efamol evening primrose oil) on fatty acid-binding proteins of rat liver

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Key words: rat liver, fatty acid-binding proteins, linoleic acid

Summary

We have studied the effects of Efamol evening primrose oil (EPO) on fatty acid-binding proteins (L-FABP) of rat liver. EPO contains 72% cis-linoleic acid and 9% cis-gamma linolenic acid. EPO has been clinically used for treatment of a number of diseases in humans and animals. EPO is also known to lower cholesterol level in humans and animals. Feeding of an EPO supplemented diet to rats (n = 9) for 2 months decreases the oleate binding capacity of purified L-FABP of rat liver whereas the palmitate binding activity was increased by 38%. However, EPO feeding did not alter the L-FABP concentrations significantly as measured by using the fluorescence fatty acid probe, dansylamino undecanoic acid. Endogenous fatty acid analysis of L-FABPs revealed significant qualititative and quantitative changes in fatty acid pattern after EPO feeding. EPO feeding decreased the endogenous palmitate level by 53% and oleate level by 64% in L-FABPs and also EPO feeding decreased the total endogenous fatty acid content from 62 nanomole per mg of protein to 42 nanomole per mg of L-FABP (n = 3).

Introduction

Dietary polyunsaturated fatty acids lower the cholesterol, triglyceride and free fatty acid levels in humans and animals [1–3]. Efamol evening primrose oil (EPO) has also been shown to be effective in lowering plasma cholesterol in humans and animals [1, 3]. One of the mechanisms of the hypocholesterolemic effect of EPO may be the facilitation of esterification and disposal of plasma cholesterol by providing desaturated metabolites of gamma linolenic acid (GLA) [3]. The formation of desaturated metabolites of GLA tend to be reduced by high cholesterol levels [2]. The metabolic effects of EPO are thought to be partially due to its high content of GLA, a precursor of longer chain desaturated essential fatty acid and of prostaglandin E_1. Prostaglandin E_1 activates cholesterol ester hydrolase and thus helps in cholesterol disposal [4]. But no investigations have been performed with regard to the effects of EPO on fatty acid-binding proteins (FABPs). Fatty acid-binding proteins are thought to be important in fatty acid transfer from plasma into various tissues as well as in the subsequent utilization, esterification, compartmentation or beta oxidation of the fatty acids [5]. FABPs (M.W. 14–15 K dalton) are cytosolic proteins present in the liver, heart, intestines and other tissues of many species [6]. The FABPs are now recognized as products of an ancient gene family, four of whose members are involved in retinoid metabolism and of whom six show high affinity for long chain fatty acids in such tissues intestinal (I-FA BP), heart (H-FABP) and liver (L-FABP), etc. (for details see ref. 7). In addition to their fatty acid-binding properties, L-FABPs also bind heme,

prostaglandin E_1 and lipoxygenase products (12-HPETE, 5-HPETE, 12-HETE, 5-HETE etc.) of arachidonic acid [8–10]. L-FABP functions also include stimulation of normal or carcinogen-induced proliferations of hepatocytes in rat liver. Bassuk *et al.* have recently shown that the concentration of L-FABP varies with the growth activity of both normal and malignant hepatocytes [11]. L-FABP concentration and its fatty acid-binding activity are also subject to modulation by several factors including hormones [12], hypolipidemic drugs such as clofibrate, tiadenol [13, 14] and dietary fat [15, 16] and also by choline deficiency [17]. Changes in the concentrations of L-FABPs correlate closely with the rates of uptake and utilization of fatty acids by the liver [12, 14].

Here we report the effect of EPO (5%, w/w) feeding on rat liver L-FABP activity.

Materials and methods

Materials. [1-^{14}C]palmitic acid (51 mCi/mmol), [1-^{14}C]oleic acid (53 mCi/mmol) and [5, 6, 8, 9, 11, 12, 14, 15-^{3}H(n)]arachidonic acid (83.6 Ci/mmol) were obtained from New England Nuclear, Boston, MA, 11-(Dansylamino) undecanoic acid was from Molecular Probes, Junction City, OR., Lipidex-1000 from Packard Instrument Co., Downers Grove, IL. Efamol evening primrose oil contains 72% cis linoleic acid and 9% gamma linolenic acid. Seeds of wild varieties of the evening primrose (*Oenothera biennis*) contain amounts of gamma-linolenic acid ranging about 2–12%. Efamol is the seed oil from varieties selected to give oil of constant quality and composition and has been extensively used in clinical trials. All other chemicals used in this study were of analytical grade quality.

Animals and diet. Female Sprague-Dawley rats (body weight, 110–140 g) were housed individually in a controlled environment, providing constant room temperature and humidity with a dark period from 2000 to 0800 h. The animals in groups (n = 9) were fed ad libitum a commercial diet or a diet containing Efamol oil (5%, w/w). The animals had unrestricted access to food and water throughout the experimental period. The rats received the control diet for at least one week before they were randomly selected for feeding on the experimental diet. Eighteen rats were divided into two groups, one group (n = 9) was fed a regular diet for 2 months and another group (n = 9) the regular diet plus Efamol oil for 2 months (for fatty acid analysis n = 3). The animals were killed and livers were removed for further studies.

Preparation of L-FABP. L-FABP was prepared essentially following the same procedure as described previously [9]. Typically, the livers were excised, blotted, weighed, perfused with ice-cold saline, and homogenized (25%, w/v) in 0.01 M phosphate buffer pH 7.4, containing 0.24 M sucrose and 1 mM EDTA. The homogenate was centrifuged twice for 10 min at 700 × g and then at 5000 × g respectively. The floating fatty layer was removed after each centrifugation. The supernatant was then again centrifuged at 105,000 × g for 90 min. Supernatant prepared from each liver was divided into two fractions: one fraction was treated to partially purify L-FABPs by ammonium sulfate treatment [17] and was then used to quantify the L-FABP content by a fluorimetric titration method while the other portion was used to purify the L-FABP. The preparations were used to determine L-FABP content by fluorescence enhancement. The final preparation was kept at − 70° C for further studies.

Purification of L-FABP. L-FABPs from the livers of control and experimental rats was purified to homogeneity using a combination of gel exclusion and anion exchange chromatography as previously described [9, 17]. Purified L-FABP was used to determine the fatty acid-binding activity and also for the determination of endogenous fatty acids contents.

Assay of L-FABP by fluorescence enhancement. L-FABP was quantitated by a fluorimetric method using the identical procedure described previously [17]. Fluorescence measurements were carried out in a Perkin-Elmer Luminescence spectrophotometer LS-5 (Perkin-Elmer) at 22° C. Fluorescence

intensity was measured at 500 nm after excitation at 350 nm, with slit width of 5 nm for both excitation and emission. Maximum fluorescence enhancement was determined for aliquots of the desalted 70% ammonium sulfate fractions. L-FABP concentrations in various samples were estimated from the maximal fluorescence enhancement of dansylamino acid. Quantitation of L-FABPs by the fluorimetric titration method was verified by the spectrophotometric method of Whitaker and Granum [18]. Bradford's method [19] was used to estimate the total protein concentration of samples in various phases of the work.

Ligand binding studies. The fatty acid-binding activity of purified L-FABP was determined using the Lipidex method [20]. L-FABP of control or experimental rat livers (20–60 μg) was incubated with radiolabeled fatty acid (1–1.6 μM) for 30 min in 50 mM sodium phosphate buffer, pH 7.4, in a total volume of 0.3 mL at room temperature. After the incubation, the vials were cooled in an ice bath. Unbound fatty acids were removed from the solution by adding 0.05 mL of an ice cold, continuously stirred Lipidex buffer suspension (1 : 1, v/v) and incubated for anthoter 10 min at 0° C. Fatty acid-binding was calculated from the amount of radioactivity present in the supernatant after centrifugation of the vials and expressed as picomoles per μg of L-FABP. Radioactivity was counted in a liquid scintillation spectrometer (Beckman LS 3155).

Fatty acid analysis. Tissue lipids were extracted by the method of Folch *et al.* [21]. Total phospholipids were separated from neutral lipids. Fatty acid analysis of different lipid fractions was performed by preparing methyl esters with BF3-methanol and analyzed by gas chromatography as described [22]. Endogenous fatty acid analysis of L-FABP was also performed following the same procedure, (n = 3).

Results

Table 1 shows the weights of rat livers and the cytosolic concentrations of L-FABPs in different experimental conditions. EPO feeding did not change either the weights of livers or the L-FABPs concentration.

We then purified the fatty acid-binding proteins from control and EPO fed rat livers. The purified protein was used to determine its fatty acid-binding capacity. Table 2 shows the effects of EPO feeding on binding activity of L-FABPs for various fatty acids. Feeding of EPO increased the palmitic acid-binding activity by 38% ($p < 0.0001$) whereas oleic acid-binding activity was decreased about 29.5% ($p < 0.0001$). There was no change in arachidonic acid binding activity of L-FABPs.

Table 3 shows the fatty acid analysis of total phospholipids, triglycerides and cholesterol ester in livers of control and experimental rats, (n = 3). In phospholipid fractions, EPO feeding had no

Table 1. Rats were fed control or diets containing 5% (w/w) EPO for two months. Livers were excised and weighed. L-FABP fractions were isolated from the livers and were quantified by fluorescence enhancement as described in the text. Values are the means ± S.D. (n = 9). Statistical differences between the control and the Efamol group were assessed by Student's t test

Treatment	Weight of liver (gm)	L-FABP concentration (μg per mg of cytosol protein)
Control[1]	10.8 ± 2.4	23.5 ± 2.3
Efamol EPO	10.1 ± 1.7*	25.0 ± 1.1[+]

* Not significant relative to control[1].

Table 2. The experimental details were the same as described in Table 1. L-FABP was purified from experimental and control rat livers. Fatty acid fatty binding activity of L-FABPs was determined as described in the text. Values are the means ± S.D. (n = 9). Statistical analysis as in Table 1

Treatment	Fatty acid binding activity		
	Oleate	Palmitate	Arachidonate
	(pico mole bound per μg of L-FABP)		
Control[1]	91 ± 6.7	78 ± 6.1	73 ± 8.4
Efamol EPO	64 ± 4.9*	107 ± 10.5*	71 ± 4.3[+]

* $p < 0.0001$ relative to control[1].
[+] Not significant relative to control[1].

influence on stearic acid or linoleic acid levels whereas it increased the levels of arachidonic acid; oleic acid and palmitic acid level were lowered compared to controls. In the cholesterol ester fraction EPO feeding increased linoleic acid by 2.5 fold and arachidonic acid by 1.85 fold but decreased palmitic acid level. The changes in the triglyceride fraction as a result of EPO feeding were very significant for most fatty acid levels. Oleic acid, palmitic acid and stearic acid levels were significantly reduced whereas the levels of linoleic acid, gamma linolenic acid and dihomogamma linolenic acid increased after EPO feeding.

Table 4 shows the endogenous fatty acid composition of L-FABP of control and EPO fed rats, (n = 3). Endogenous levels of palmitic acid and oleic acid were reduced by 53% and 63% respectively after EPO feeding when compared with control. But EPO feeding increased the levels of gamma linolenic acid, dihomogamma linolenic and arachidonic acid significantly. There were no significant changes in the levels of stearic acid and linoleic acid.

Discussion

Efamol evening primrose oil (EPO) has been shown to be effective in lowering plasma cholesterol [1, 4]. Table 3 shows that EPO feeding significantly increased the levels of polyunsaturated acids and decrease the palmitate level in the cholesterol ester fraction of liver lipids. This observation is in agreement with the previous report from our Institute. The mechanism of the EPO effect on the cholesterol level is not fully understood but it increases the polyunsaturated fatty acids in the cholesterol ester fraction and thus facilitates cholesterol disposal.

This paper demonstrates that EPO feeding changes the behavior of the L-FABP which is believed to play an important role in intracellular fatty acid utilization. Transport of fatty acids derived from the plasma and their utilization either by beta oxidation in mitochondria or by esterification in smooth endoplasmic reticulum may require the participation of fatty acid-binding proteins. The fatty acid compositions of cell membranes and other cellular components are likely to be determined both by dietary intake and by differential metabolism of fatty acids. L-FABP behavior is likely to be important in such differential metabolism. In addition, arachidonate (20 : 4n6) or dihomogamma linolenic acid (20 : 3n6) bound to some FABPs serve as precursors of a large number of locally acting hormones including prostaglandins and thromboxanes and leukotrienes. Recent observations that PGE1 and 15-HPETE/5-HPETE (immediate precursor of LTB4 and LTC4) bind to the same binding sites in L-FABP in a competitive manner suggest that one may influence other's synthesis by modulating L-FABP binding activity [9,

Table 3. Fatty acids in liver phospholipids, Cholesterol ester (CE) and triglycerides. Results are expressed as gm/100 gm of total fatty acid present. Each value is the mean ± S.D. (n = 3). Statistical analysis was done by Student's t test. Regular Chow (RC), Regular chow + 5% EPO (EPO)

Fatty acids	RC	EPO	RC	EPO	RC	EPO
	Phospholipids		Cholesterol ester		Triglycerides	
18 : 2n6	16.4 ± 0.9	16.7 ± 1.4	5.2 ± 0.8	13.0 ± 3.3[b]	24.2 ± 2.8	42.6 ± 2.9[c]
18 : 3n6	0.17 ± 0.1	0.4 ± 0.1[c]	N.D.	0.56 ± 0.1[b]	N.D.	2.1 ± 0.6[c]
20 : 3n6	1.3 ± 0.2	1.1 ± 0.2	N.D.	N.D.	N.D.	1.8 ± 0.2[c]
20 : 4n6	27.6 ± 1.9	36.3 ± 1.2[c]	3.5 ± 1.7	6.5 ± 2.1[a]	2.6 ± 1.2	6.8 ± 1.8[c]
18 : 1n9	5.3 ± 1.6	4.1 ± 0.4[b]	8.7 ± 2.1	9.7 ± 2.0	28.8 ± 2.1	14.2 ± 1.4[c]
18 : 0	17.4 ± 1.6	16.7 ± 1.4	9.9 ± 0.7	11 ± 1.5[c]	3.5 ± 0.8	2.3 ± 0.7[b]
16 : 0	12.9 ± 1.1	11.5 ± 0.3[a]	47.2 ± 4.2	40 ± 5.6[a]	24.2 ± 2.1	17.9 ± 1.7[c]

[a] $P < 0.02$; [b] $P < 0.05$; [c] $P < 0.0001$.

10]. Fatty acid-binding protein stimulates desaturation of stearate to oleate by virtue of its higher affinity for oleate compared to stearate [23]. It has been reported that an increase in fatty acid-binding protein occurs in rat liver after feeding high fat and carbohydrate diets [15]. But Herzberg and Rogerson [16] reported that feeding rat a diet rich linoleic acid led to a 30% fall in liver fatty acid-binding protein, whereas a palmitate-rich diet had no effect on L-FABP. It is therefore not yet well defined as to how various dietary fats modulate L-FABP and its binding activity. We have now found that feeding of EPO increases the palmitate binding of L-FABP by 38% whereas it decreases oleate binding activity without changing L-FABPs concentration. The effects of EPO, which contains gamma-linolenic acid as well as linoleic acid, are therefore different from those of linoleic acid alone. This suggests that different fatty acids may have very different effects on L-FABP function. EPO feeding increases palmitate binding activity of L-FA BPs thereby enhancing the utilization of palmitate by beta oxidation [24] or palmitoylation of various proteins. The EPO effects suggests that the relative rates of oxidation of saturated and monounsaturated fats may be modulated by the diet could have important implications for cardiovascular health and for regulation of body weight, but understanding of what those implications are will require much more work. The effects of EPO on rat liver L-FABP are similar to those of clofibrate feeding in rat heart [25]. The effects of EPO on L-FABP are different from those observed with other fat diets [14, 15], probably because of its unique fatty acid composition.

Our observations suggest new routes of investigation of the effects of specific dietary fats.

Table 4. Endogenous fatty acids composition of L-FABPs isolated from control and EPO fed rat livers. Each value is the mean ± S.D. (n = 3). Statistical analysis was done by Student's t test. Regular chow (RC), Regular chow + 5% EPO (EPO)

Fatty acids	RC	EPO
	(nmole/mg of protein)	
16:0	16.46 ± 1.6[a]	8.72 ± 1.45[a]
18:0	5.33 ± 0.37	4.49 ± 0.69[c]
18:1n9	15.76 ± 1.67	5.91 ± 0.69[a]
18:2n6	13.90 ± 1.88	14.72 ± 1.18
18:3n6	0.53 ± 0.14	0.89 ± 0.15[b]
20:3n6	N.D.	0.58 ± 0.09[a]
20:4n6	4.33 ± 1.66	6.08 ± 0.62[c]

[a] $P < 0.0005$; [b] $P < 0.005$; [c] $P < 0.05$.

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Quantitation of plasma membrane fatty acid-binding protein by enzyme dilution and monoclonal antibody based immunoassay

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Key words: fatty acids, fatty acid-binding protein(s), hepatocyte, immunoassay, plasma membranes, transport

Summary

A plasma membrane fatty acid-binding protein (h-$FABP_{pm}$) has been isolated from rat hepatocytes. Analogous proteins have also been identified in adipocytes, jejunal enterocytes and cardiac myocytes, all cells with high transmembrane fluxes of fatty acids. These 43 kDa, highly basic (pI = 9.1) $FABP_{pm}$'s appear unrelated to the smaller, cytosolic FABP's (designated FABP's) identified previously in the same tissues. h-$FABP_{pm}$ appears closely related to the mitochondrial isoform of glutamic-oxaloacetic transaminase (mGOT), and both the purified protein and liver cell plasma membranes (LPM) possess GOT enzymatic activity. From their relative GOT specific activities it is estimated that h-$FABP_{pm}$ constitutes approximately 2% of LPM protein, or about 0.7×10^7 sites per cell. A monoclonal antibody-based competitive inhibition enzyme immunoassay (CIEIA) for h-$FABP_{pm}$ is described; it yields an estimate of 3.4×10^7 h-$FABP_{pm}$ sites per hepatocyte. Quantitated by either method, h-$FABP_{pm}$ appears to be a highly abundant protein constituent of LPM.

Introduction

Long chain free (non-esterified) fatty acids (FFA) have long been thought to enter cells passively [1, 2], perhaps by simple partitioning into the lipid bilayer [3]. However, studies over the past decade in several laboratories have established that the uptake of FFA into hepatocytes [4, 5], adipocytes [6–8], cardiac myocytes [9, 10] and jejunal enterocytes [11] has the kinetic properties of carrier mediated transport. Work in the authors' laboratory has led to the isolation and partial characterization of basic (pI = 9.1) 43 kDa plasma membrane fatty acid-binding protein(s) ($FABP_{pm}$'s) from each of the cell types enumerated [8, 9, 12–14]. $FABP_{pm}$'s isolated from liver, gut, adipocytes and cardiac myocytes are immunologically cross-reactive [14], and are closely related if not identical. Moreover, the hepatic $FABP_{pm}$ has, surprisingly, been found to have a close structural and immunologic similarity to the mitochondrial isoform of the enzyme glutamic-oxaloacetic transaminase (mGOT), and to possess GOT enzymatic activity [15]. By contrast, these $FABP_{pm}$'s are both structurally and immunologically distinct from the cytosolic fatty acid-binding proteins ($FABP_c$'s)[1] identified previously in the same tissues [12, 13, 16]. The various

[1] As previously proposed [14], FABP's are designated with prefixes h-, g-, a-, my-, or sm-, to indicate origin from hepatic, gut, adipose, myocardial or skeletal muscle tissue, and a subscripted suffix $_c$ or $_{pm}$ to denote cytosolic or plasma membrane subcellular localization. Thus, h-$FABP_{pm}$ and g-$FABP_c$ denote hepatic plasma membrane and gut cytosolic FABP's, respectively.

$FABP_{pm}$'s play an important, if thus far ill defined, role in FFA transport, as indicated by the fact that polyclonal antisera directed against these proteins selectively and non-competitively inhibit cellular FFA uptake [5, 8–11].

In the present report we describe the development of a competitive inhibition enzyme immunoassay for the quantitation of $FABP_{pm}$'s both in solution and on the surface of cells, employing a monoclonal anti h-$FABP_{pm}$. The results, confirmed by an independent enzyme dilution technique, demonstrate that $FABP_{pm}$'s are highly abundant surface proteins in cells with high transmembrane fatty acid fluxes.

Experimental procedures

Preparation of liver plasma membranes and cell suspensions

Sinusoidally enriched liver plasma membranes (LPM) were prepared and their purity assessed as previously described [12, 13, 17, 18]. Isolated single cell suspensions of rat hepatocytes and adipocytes, and canine cardiac myocytes were prepared by established methods, and in each instance met viability criteria employed in previous studies from our laboratory [4, 5, 8, 9].

Purification of h-$FABP_{pm}$

h-$FABP_{pm}$ was isolated from LPM and purified to homogeneity by the method of Potter *et al.* [14], which involves preparative isoelectric focusing, oleate-agarose affinity chromatography, and high performance liquid chromatography over gel permeation and/or hydrophobic interaction matrices. Purity of each preparation was assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting, employing a monospecific rabbit anti rat h-$FABP_{pm}$ [14], and invariably exceeded 98%.

Enzyme assays

The rate of formation of oxaloacetate from 2-oxoglutarate as a measure of GOT enzymatic activity was determined by coupling this reaction with the reduction of the product by an excess of malic dehydrogenase in the presence of NADH. The oxidation of NADH was determined from the decrease in absorbance at 340 nm. A kit (Sigma Diagnostics, procedure 58-UV) was employed for this determination. When measuring mGOT activity in LPM, Triton X-100 was first used to solubilize the proteins [19]. One unit of enzymatic activity was defined as the amount of enzyme which produced 1 μmol of oxaloacetate per min at 30° C. Total protein in h-$FABP_{pm}$ preparations and LPM was measured by the Lowry procedure [20] as standardized against bovine serum albumin (BSA).

Production and characterization of monoclonal antibodies

Methods for the production, screening and characterization of anti h-$FABP_{pm}$ monoclonal antibodies will be reported in detail elsewhere.[2] Briefly, female Balb/c mice (Jackson Laboratories, Bar Harbor, ME) were immunized with 25 μg of purified h-$FABP_{pm}$ in complete Freund's adjuvant; and boosted intra-peritoneally every 14 days. The final boost was administered 3 days prior to cell fusion. For the production of hybridoma lines, spleen cells were harvested aseptically from immunized animals and fused with mouse NS-1 myeloma cells, using 50% w/v PEG (carbowax 4,000/5% DMSO) [21]. The resulting hybridoma lines were screened initially by a conventional ELISA procedure employing 96 well microtiter plates pre-coated with known quantities of purified h-$FABP_{pm}$. Positives were subjected to 3 rounds of limiting dilution to ensure monoclonality, and then grown in ascites in female Balb/c mice pretreated with pristane. Ascitic fluid was collected and stored at − 70° C. Secondary screening was performed using isolated rat

[2] Zhou S-L, Potter BJ, Stump D, Sorrentino D and Berk PD. Manuscript in preparation.

hepatocytes fixed in 2% paraformaldehyde as target cells and either FITC-conjugated or peroxidase-conjugated goat anti-mouse immunoglobulins (Sigma) for immunofluorescence or immunohistochemical staining, respectively. Specificity of the monoclonal antibodies was further assessed by SDS-PAGE of native and denatured samples of purified h-$FABP_{pm}$ or Triton X-100 extracts of LPM, followed by Western immunoblotting with the monoclonal antibody in question. Denatured samples were prepared by boiling for 3 min in the presence of dithiothreitol (DTT) and SDS. The ability of the monoclonal antibodies to inhibit the uptake of [^{3}H]-oleic acid by isolated hepatocyte suspensions was examined as previously described for rabbit anti rat h-$FABP_{pm}$ polyclonal antisera [5]. The subisotypes of the various monoclonal immunoglobulins produced were determined by means of a subisotyping kit (Hyclone Laboratories).

Competitive inhibition enzyme immunoassay

A competitive inhibition enzyme immunoassay (CIEIA) for h-$FABP_{pm}$ was developed employing 96 well microtiter plates incubated at 4° C overnight with 50 μl per well of purified h-$FABP_{pm}$ (2 mg/ml in phosphate buffered saline [PBS], pH 7.4) as solid phase adsorbent. The fluid phase of the competitive inhibition reaction was set up in separate plates, blocked at 37° C for 1 hour with 10% BSA, and consisted of 50 μl of test sample containing h-$FABP_{pm}$ and 50 μl of monoclonal anti h-$FABP_{pm}$ antibody. After mixing, aliquots of the sample and monoclonal antibody were transferred to the antigen-coated plate, incubated at room temperature for 1 hour, and then washed 5 times in 0.05% Tween 20/PBS. Fifty μl of alkaline phosphatase conjugated anti-mouse immunoglobin diluted 1 : 1000 in PBS-surfactant (Hyclone) was added to each well and incubated for an additional 1 hour at room temperature. After washing a further 5 times as described above, 100 μl of substrate solution (1 mg/ml p-nitrophenyl phosphate in 0.1 M ethanolamine buffer, pH 9.6) was added and incubated for a final 30 min at room temperature. Reaction products were measured spectrophotometrically at 405 nm. Serial dilutions of standards (purified h-$FABP_{pm}$, 30 μg/ml, or isolated rat hepatocytes, 1×10^7/ml) in 0.5% BSA/PBS were analyzed in quadruplicate to generate standard curves.

Results

Screening and characterization of monoclonal antibodies

More than 300 wells resulting from 2 separate fusions were screened by the initial ELISA assay; 30 wells were positive and 13 of these have thus far been cloned by 3 rounds of limiting dilution and grown in ascites, achieving titers ranging from 8×10^3 to 3×10^5. Of the 13 monoclonal antibodies examined thus far, 11 were IgM, and 2, designated 5A1 and 5C2, were IgG_3. Nine produced staining of rat hepatocyte plasma membranes in indirect immunofluorescence and/or immunohistochemical assays. Eleven of the 13 monoclonals examined were positive on Western blotting against either native or denatured h-$FABP_{pm}$, and/or identified a 43 kDa band on Western blotting after SDS-PAGE of Triton X-100 extracts of rat LPM. Three of these, designated 4A4, 5C2 and 5D4, were evaluated further in dot immunobinding, immunoprecipitation and competitive ELISA assays, which established their specificity for h-$FABP_{pm}$. Only one monoclonal, 4A4, consistently inhibited uptake of [^{3}H]-oleate by isolated hepatocyte suspensions; it was accordingly, selected for use in the CIEIA. Full details of the characteristics of all 13 monoclonal antibodies investigated will be reported elsewhere.[2]

Quantitative immunoassay of h-$FABP_{pm}$

Using highly purified h-$FABP_{pm}$ as a standard, the CIEIA is highly reproducible, with a coefficient of variation of $\pm$ 10%, and log-linear up to 1000 ng/well (Fig. 1). A typical inhibition curve obtained with serial dilutions of a rat hepatocyte suspension is illustrated in Fig. 2. By comparison of the 50%

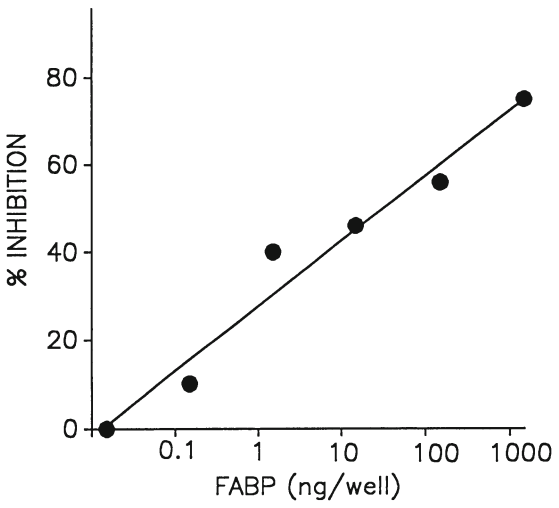

Fig. 1. Competitive inhibition enzyme immunoassay for h-$FABP_{pm}$. Standard curve was constructed from serial dilutions of a highly purified protein sample.

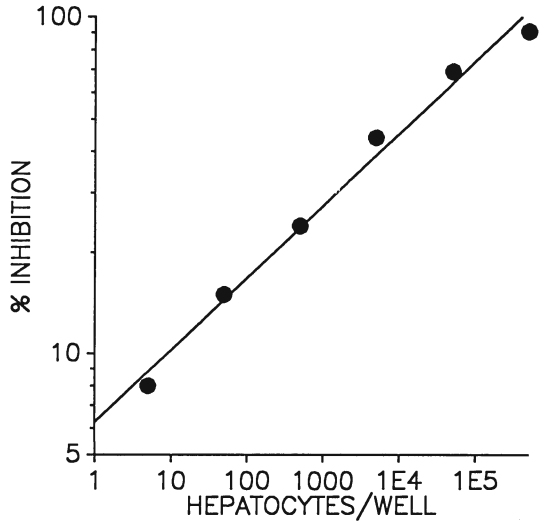

Fig. 2. Competitive inhibition enzyme immunoassay for h-$FABP_{pm}$. Serial dilutions of an isolated rat hepatocyte preparation.

inhibition points in the two curves, and using an estimated molecular weight of 43,000 for h-$FABP_{pm}$, we arrive at estimates of 1–6 × 10^7 (average 3.4 × 10^7) h-$FABP_{pm}$ molecules exposed on the surface of each hepatocyte. Some of the variability observed between hepatocyte preparations may reflect differences in the ages and dietary histories of the animals studied. Highly preliminary further studies suggest that there is no significant difference between male and female rat hepatocytes in the number of exposed h-$FABP_{pm}$ molecules per cell, but that isolated adipocytes and cardiac myocytes have approximately 10 times more sites per cell than do hepatocytes, in rough proportion to their relative Vmax's for FFA uptake.

Quantitation of h-$FABP_{pm}$ by enzyme dilution

The average GOT specific activity of LPM preparations is 2.8 U/mg of LPM protein, while that of purified h-$FABP_{pm}$ is approximately 140 U/mg. While the coefficient of variation for these measurements individually is ± 20%, these average values indicate that h-$FABP_{pm}$ represents approximately 2% of LPM protein. Since 1 g of rat liver contains 2.3 mg LPM protein [17] and 1 × 10^8 hepatocytes, it follows that each hepatocyte contains 23 pg of LPM protein, 2% of which (460 fg) is h-$FABP_{pm}$. For a 43 kDa protein, this represents 0.011 fmol, or 0.7 × 10^7 molecules per cell.

Discussion

Although passive transfer, perhaps by partitioning into the lipid bilayer [3] has long been considered the mechanism by which long chain FFA enter cells, recent studies suggest that, at least in cells with high transmembrane FFA fluxes such as the intestinal epithelial cell [11], the hepatocyte [4, 5], the adipocyte [6–8] and the cardiac myocyte [9, 10], at least a portion of FFA uptake occurs by a specific, saturable and phloretin inhibitable transport process. Under appropriate experimental conditions, this mechanism exhibits all of the typical features of carrier mediated transport including selective competitive inhibition between various long chain FFA, but not by other classes of organic anions such as bile acids, or bilirubin; counter transport; and the pre-loading (trans-stimulation) effect. There has been considerable controversy about a putative albumin receptor or alternative mechanisms which, at least in the liver, were said to modulate FFA uptake by facilitating dissociation of albumin: FFA complexes at the cell surface

[22–27]. However, more recent studies indicate that in isolated adipocytes and cardiac myocytes as well as hepatocytes, when physiologic concentrations of albumin are employed, FFA uptake is a saturable function of the unbound FFA concentration in the bathing medium, and uptake kinetics are fully consistent with conventional pharmacokinetic models [28]. Studies in the isolated, perfused rat liver [22, 29], isolated hepatocytes [4, 5] and sinusoidal LPM vesicles [30] all suggest that hepatocellular FFA uptake is a potential sensitive process linked in some way to sodium transport. The precise nature of the coupling of FFA and Na^+ influx remains unclear, but appears not to reflect a Na^+: FFA symport analogous to that involved in bile acid transport [31, 32]. This aspect of FFA transport remains an area of intense investigation.

Studies of the binding of FFA to isolated LPM were consistent with the existence of a single class of trypsin sensitive, high affinity fatty acid-binding sites, with $K_a = 10^{-8}$ M, on the membranes [33]. Subsequently, a discrete fatty acid-binding protein of 43 kDa was extracted and purified from isolated hepatocyte plasma membranes [12]. Analogous proteins have been isolated from the plasma membranes of adipocytes, jejunal enterocytes and cardiac myocytes [8, 9, 13, 14]. All react with monospecific polyclonal rabbit antisera against h-FA BP_{pm}; in the case of the $FABP_{pm}$'s from adipocytes and gut, the reaction yields a line of immunologic identify in agar gel diffusion studies; the cardiac myocyte protein exhibits spur formation. This and minor differences in isoelectric point suggest that the cardiac protein differs in as yet undefined ways from the other three, which appear to be strikingly similar, if not identical [14].

Initial attempts to sequence h-$FABP_{pm}$ led to the unanticipated finding that the 24 N-terminal amino acids were identical to those of the mitochondrial isoform of the well studied enzyme glutamic-oxaloacetic transaminase (mGOT) [15]. These studies indicated (i) that the overall amino acid composition of h-$FABP_{pm}$ and mGOT are strikingly similar; (ii) both proteins migrate identically on SDS-PAGE, with identical apparent molecular weights of 40,000, and through four different HPLC columns which separate proteins on the basis of quite different physicochemical properties; (iii) both proteins have the same complex pattern of highly basic charge isomers, with an average pI of 9.1, and virtually identical absorption spectra under both acid and basic conditions. Monospecific, polyclonal rabbit antisera raised against either protein gave similar patterns of immunofluorescent staining of snap frozen liver sections, identifying antigens both on the plasma membrane and in intracellular (presumably mitochondrial) sites. Either antiserum reacted with both proteins on Western immunoblotting, and both antisera selectively inhibited the uptake of [^{3}H]-FFA by isolated hepatocytes. mGOT has a definite although not yet quantified affinity for FFA, and can be separated from protein mixtures by oleate-agarose affinity chromatography. Finally, as already noted, both isolated LPM and purified h-$FABP_{pm}$ possess GOT enzymatic activity, a property exploited in the present study in an effort to quantitate the amount of this protein on the hepatocyte plasma membrane. These observations suggest that the mitochondrial enzyme mGOT and the plasma membrane protein h-$FABP_{pm}$ are closely related, if not identical [15], raising a host of interesting questions in cellular and molecular biology. We are attempting to answer these questions through the analysis of cDNA clones obtained by immunoscreening of a rat liver cDNA library.

Polyclonal antisera against h-$FABP_{pm}$ do not cross-react with cytosols containing FABPc [12–14]. Differences in size and physicochemical properties also suggest that the plasma membrane and cytosolic fatty acid-binding proteins identified in various tissues [14, 16] are not closely related. Although only very limited amino acid sequence data are available for h-$FABP_{pm}$ per se, if one accepts that it is indeed closely related to mGOT, for which the full amino acid sequence is known [34–36], this line of evidence also indicates no significant structural homology between the two types of FABP.

Its remains to explore whether $FABP_{pm}$ and FABP in a given cell type might be coordinately regulated, as appears to be the case for the plasma membrane transporter, intracellular binding proteins (glutathione-S-transferases 1-1 and 1-2) and conjugating enzyme (UDP-glucuronyl transferase)

involved in hepatic bilirubin transport [37]. Although the CIEIA for quantitation of h-$FABP_{pm}$ will be a useful tool for addressing this issue, only very fragmentary and preliminary data are available thus far. Within the liver, FABP is increased in females compared to males, and in experimental diabetes or following the administration of a high fat diet or of clofibrate [16]. Preliminary data obtained with the CIEIA suggest that there is no difference in h-$FABP_{pm}$ levels between male and female rat hepatocytes. While this is at variance with the results for FABP, it is consistent with kinetic observations indicating that sex differences in hepatocellular FFA uptake velocity reflect differences in the Km, rather than the Vmax, of the transport system due presumably to difference in membrane lipid composition between the sexes [38]. By contrast, both diabetes and a high fat diet increase the Vmax for FFA uptake [39]. We speculate that this change will be accompanied by an increase in immunoassayable h-$FABP_{pm}$, but these data are thus far incomplete. Both diabetes and fat-feeding in the rat do result in an increase in plasma FFA [39]. If the observed increases in Vmax for FFA uptake are shown to reflect increased expression of h-$FABP_{pm}$, it will suggest that h-$FABP_{pm}$ is substrate inducible. Studies of the responses of $FABP_{pm}$ and FABP to a variety of experimental perturbations are being undertaken currently, so as to establish the extent to which the responses of the two proteins are parallel or divergent.

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The membrane fatty acid-binding protein is not identical to mitochondrial glutamic oxaloacetic transaminase (mGOT)

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Summary

For evaluation whether the membrane fatty acid-binding protein is related to mGOT, studies on the structure and function of both purified proteins were performed. Physicochemical characterization revealed that both proteins are different: the membrane fatty acid-binding protein has a molecular weight of 40 kD and a pI of 8.5–9.0, whereas rat mGOT has a molecular weight of 44 kD and a pI of 9.5–10.0. According to this distinct differences, they migrated separately on 2-dimensional electrophoresis. Furthermore, monospecific antibodies against the membrane fatty acid binding protein did not react with rat mGOT. In co-chromatography studies only the membrane fatty acid-binding protein showed affinity for long chain fatty acids, but not mGOT. Moreover, membrane binding studies were performed with the monospecific antibody to the membrane fatty acid binding protein. The inhibitory effect of this antibody on plasma membrane binding of oleate was reversed after preabsorption of the antibody with the membrane fatty acid binding protein, but was not affected after preabsorption with mGOT. These results indicate that the membrane fatty acid binding protein and mGOT are structurally and functionally not related. The data also support the significance of this membrane protein in the plasma membrane binding process of long chain fatty acids.

Introduction

In previous studies from our laboratory it was shown that uptake of long chain fatty acids by cardiomyocytes [1], hepatocytes [2–5] and mucosal cells [6] reveals criteria of carrier-mediated membrane transport kinetics. From all of these organs a single plasma membrane fatty acid binding protein with a molecular weight of 40 kD and a pI of 9 was isolated employing a one-step oleate-agarose affinity chromatography procedure of solubilized total plasma membrane proteins [1, 7, 8]. With monospecific antibodies to this protein membrane binding as well as cellular influx of fatty acids was selectively inhibited, demonstrating its physiologic significance as transmembrane carrier protein [1–8]. Furthermore, studies of the responsible driving forces for cellular fatty acid uptake were performed with isolated basolateral rat liver plasma membrane vesicles [9]. It was shown that active transmembrane movement of fatty acids is Na^+-dependent and stimulated by a negative intravesicular charge [9]. The suggestion of an electrogenic Na^+-fatty acid cotransport system was supported by studies of Weisiger *et al.* [10] that showed in the isolated perfused rat liver that in presence of Na^+

oleate influx is associated with transfer of positive charge into the intracellular space. Other groups also defined kinetic criteria of a carrier mediated membrane fatty acid translocation process in isolated cardiomyocytes [11] and rat adipocytes [12–14].

Recently, Berk *et al.* reported about the identification of a membrane fatty acid binding protein prepared by a different, two-step isolation technique [15]. On SDS-PAGE this protein appeared to be similar to the originally isolated membrane fatty acid binding protein. However, more detailed analysis demonstrated homology to mitochondrial GOT [16–18]. Therefore these authors suggested that the membrane fatty acid binding protein might be, at least in part, identical to mGOT. So far we have no evidence that mGOT, a mitochondrial matrix enzyme of amino acid metabolism, has also plasma membrane fatty acid translocation or binding capacity. Therefore, we evaluated function and structure of our membrane fatty acid-binding protein – prepared by the original isolation procedure – in direct comparison to purified rat mGOT, provided by Dr. Passarella and Dr. Doonan [19]. The question was whether both proteins might be related and whether our laboratory and the group of Berk *et al.* have identical or different proteins.

Experimental procedures

Isolation of rat liver plasma membranes

Subcellular fraction enriched in plasma membranes were prepared from livers of Wistar rats by differential centrifugation [8] and characterized by marker enzym determination [9]. Membrane protein content was determined by the method of Lowry *et al.* [20].

Preparation of the plasma membrane fatty acid binding protein

Pooled plasma membranes with 500 mg membrane proteins were solubilized with 1% (v/v) triton X100 in 1 mM $NaHCO_3$ (pH 7.4) for 2 h at 4° C [8]. After centrifugation at 100.000 g for 60 min the clear supernatant was applied to a Bio-Beads SM-2 column to remove excess of detergent. The solubilized membrane protein mixture was then loaded at a flow rate of 30 ml/hr onto a 60 ml oleate-agarose affinity chromatography column, prepared by the method of Peters *et al.* [8, 21]. Proteins which were unspecifically retained to the hydrophobic matrix of the column were washed out with 1.21 0.5 M NaCl/0.02 M phosphate buffer until no further protein appeared in the effluent. Bound protein was eluted with 8 M urea, and immediately thereafter dialyzed with 0.15 M NaCl/0.02 M phosphate buffer, pH 7.4, (PBS) to remove the urea. Recovered proteins were then concentrated by ultrafiltration (Diaflow PM-10 membranes, Amicon), before further characterization was performed.

Characterization of proteins

Proteins were characterized by SDS-PAGE on 12.5% gels [22] using the silver stain kit from Amersham (Amersham, UK). Analytical isoelectric focusing was performed as before [8]. For two-dimensional electrophoresis the proteins were separated in the first dimension over a pH range of 3.5–10 according to O'Farrell [23] and in the second dimension according to Laemmli in a 12.5% gel [24]. Gels were stained with Coomassie blue [25].

Co-chromatography studies

Aliquots of the purified membrane fatty acid binding protein and mGOT as well as albumin and a defined Bence Jones protein (provided by the Max Planck Institute in Göttingen, FRG) as positive and negative controls, respectively, were incubated with tracer amounts of [^{3}H]oleate in 0.1 M NaOH, neutralized with 0.1 M HCL and incubated in PBS for 30 min at room temperature. Samples containing 5 μg protein in a total volume of 50 μl were subjected to gel filtration over an UltroPac TSK G3000 SW HPLC column (LKB/Pharmacia, Freiburg, FRG), equilibrated with 0.1 M NaCl, 0.1 M Na-phosphate (pH 6.8). Elution was carried out with the same buffer at 0.25 ml $\times$ min^{-1}. Eluted fractions were monitored for protein (OD at 210 nm) and radioactivity [26].

Immunoblot analysis

Protein samples were applied to two-dimensional electrophoresis (see above) or to SDS-PAGE using

5–20% gradient gels and run at 10 mA per gel over 12 hours. After blotting onto nitrocellulose sheets (Schleicher & Schüll, Dassel, FRG) antiserum to the membrane fatty acid binding protein [8] or preimmune serum as control was added, both in dilutions of 1 : 200 with PBS [27]. A peroxidase coupled swine anti-rabbit immunoglobulin (Dakopatts, Hamburg, FRG) (1 : 200 diluted in PBS) served as second antibody. For staining 120 mg 4-chloro-1-naphthol (Sigma, München, FRG) and 150 μl 30% (v/v) H_2O_2 in 200 ml 20% (v/v) methanol/Dulbecco's phosphate buffered saline was used [28].

Furthermore an Ouchterlony radial double-immunodiffusion test on agar plates was performed [29].

Studies of [^{3}H]oleate binding to isolated rat liver plasma membranes

In these experiments the effect of mGOT and the membrane fatty acid binding protein on the membrane binding process of [^{3}H]oleate was analyzed. Rat liver plasma membrane aliquots (250 μg protein in 250 μl PBS) were pretreated with 20 μg (in 250 μl PBS) of the IgG-fraction [30] of the same rabbit antiserum to the membrane fatty acid binding protein which in previous studies showed inhibition of membrane binding and cellular uptake of [^{3}H]oleate [3–8]. In control experiments the IgG-fraction of the preimmune serum was used. For determination whether the membrane fatty acid binding protein and mGOT are identical and are involved in the membrane binding process of fatty acids, the antibodies (20 μg IgG) were preabsorbed with 10 μg of both proteins. For these experiments, antibodies and proteins were preincubated in 250 μl PBS for 2 h at room temperature before they were added to the plasma membrane aliquots (250 μg protein in 250 μl PBS) and incubated for 5 h. Thereafter 500 μl 120 μM [^{3}H]oleate : albumin (2 : 1) were added in PBS and incubated at room temperature for 30 min, as described earlier [8]. Membrane binding was determined by vacuum filtration [1].

Statistical analysis

Results are given as means ± S.D. The t-test was used to test for significant differences among

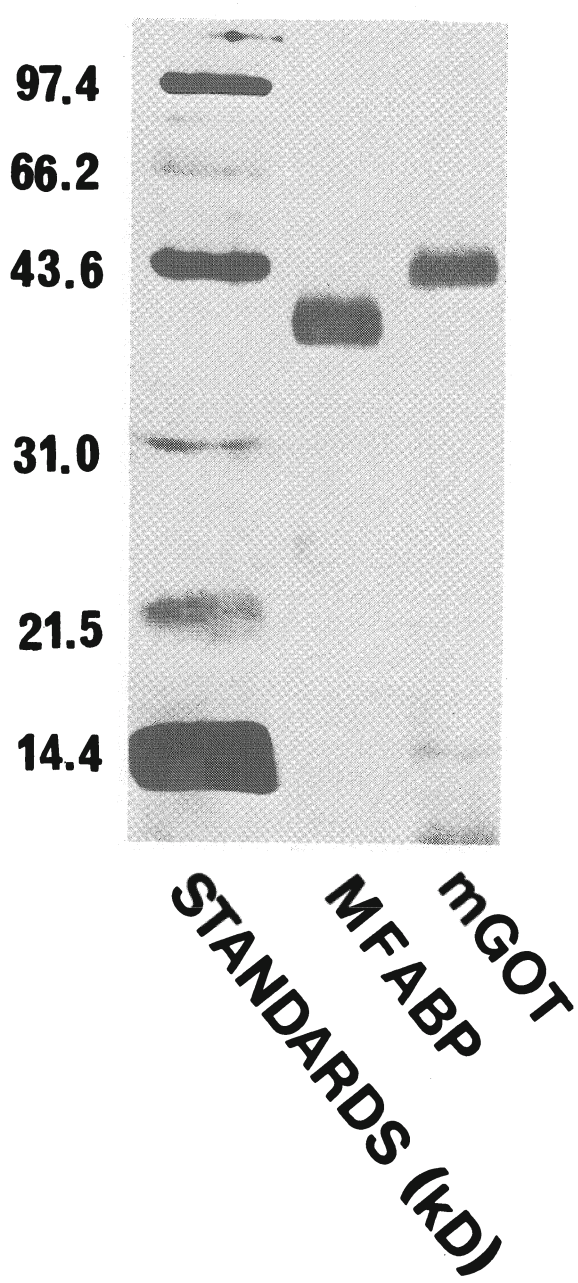


Fig. 1. SDS-PAGE analysis of the membrane fatty acid-binding protein (MFABP) prepared from triton X100 solubilized rat liver plasma membranes by oleate-agarose affinity chromatography. For comparison purified rat mGOT is applied in the right lane. Molecular weight standards are shown on the left.

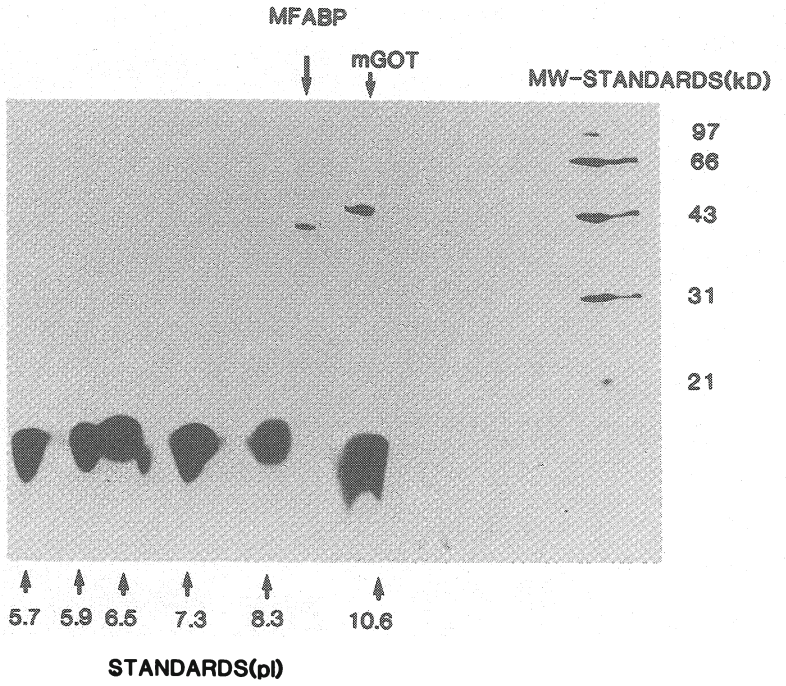


Fig. 2. Two-dimensional electrophoresis of the membrane fatty acid-binding protein and mGOT (1. dimension IEF (pH 3.5–10); 2. dimension 12.5% SDS-PAGE). Both proteins and IEF standard proteins were simultaneously applied. The membrane fatty acid-binding protein (MW 40 kD, pI 8.5–9.0) is distinct from rat mGOT (MW 44 kD, pI 9.5–10.0). A Coomassie blue staining procedure was used.

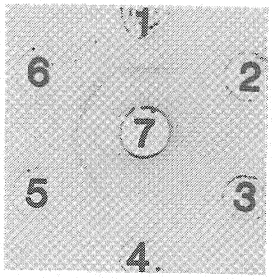
means [31]. P-values equal to or less than 0.05 were considered significant.

Results

The membrane fatty acid binding protein was isolated from triton X100 solubilized rat liver plasma membrane proteins by a one step oleate-agarose affinity chromatography separation procedure [8]. Proteins which were unspecifically retained to the hydrophobic matrix of this affinity chromatography column were removed by extensive washing with 20 column volumes of high salt buffer. Thereafter, the column was eluted with 8 M urea and the recovered proteins dialyzed against 0.15 M NaCl/0.02 M phosphate buffer (pH 7.4) (PBS). This membrane fatty acid binding protein was directly compared to purified mGOT, provided by Dr. Passarella and Dr. Doonan. Silver staining of gels after SDS-PAGE showed single proteins indicating the relative purity of the preparations. The apparent molecular weight of both proteins were different. While mGOT showed a molecular weight of 44 kD, the membrane fatty acid binding protein appeared at 40 kD (Fig. 1).

By isoelectric focusing also single protein spots of both proteins were identified revealing distinct pI-values of 9.5–10.0 and 8.5–9.0 for mGOT and the membrane fatty acid binding protein, respectively. Moreover, when both proteins were simultaneously applied to two-dimensional electrophoresis two separate protein spots were detected (Fig. 2). Based on this physicochemical characterization it is obvious that both proteins are different.

Next it was evaluated whether mGOT and the membrane fatty acid binding protein may share any antigenic determinants. Therefore, an Ouchterlony double immunodiffusion test with a monospecific polyclonal rabbit antibody to the membrane fatty acid binding protein was performed. This antibody was the same which in previous studies inhibited



1. MFABP
2. m GOT
3. rat serum
4. rat liver cytosol
5. rat liver homogenate
6. sinusoidal plasma membrane of rat liver

Fig. 3. Ouchterlony double radial immunodiffusion analysis of various protein preparations against the monospecific antiserum to the membrane fatty acid binding protein. The antibody reacted with the membrane fatty acid binding protein (MFABP), rat liver homogenate and rat liver plasma membrane proteins. No reaction was observed with rat mGOT, rat serum and rat liver cytosol.

membrane binding and hepatocellular uptake of long chain fatty acids [3–8]. A precipitin line was detectable between this antibody and the membrane fatty acid binding protein, rat liver plasma membrane proteins and rat liver homogenate. There was no reaction with mGOT, rat liver cytosol and rat serum (Fig. 3). This was confirmed by the highly sensitive Western blot analysis. The same antibody reacted with the membrane fatty acid binding protein, rat liver plasma membranes and rat liver homogenate detecting a 40 kD protein, but not with mGOT (Fig. 4). In a two-dimensional immunoblot with the protein mixture of isolated rat liver plasma membranes this antibody detected a single protein with a molecular weight of 40 kD and a pI of 9 which is identical to the authentic membrane fatty acid binding protein, but different to mGOT.

Since there was no apparent homology of the membrane fatty acid binding protein and rat mGOT, the question was whether mGOT has any fatty acid binding capacity. In co-chromatography studies with both proteins and tracer amounts of [^{3}H]oleate, the membrane fatty acid binding protein co-eluted together with radiolabeled fatty acid, whereas mGOT eluted separately from any [^{3}H]oleate under identical experimental conditions.

The suggestion that mGOT is not directly involved in the plasma membrane binding and translocation process of long chain fatty acids, was derived from antibody inhibition studies. First it was established that the IgG-fraction of the monospecific antiserum to the membrane fatty acid binding protein inhibited binding of [^{3}H]oleate to rat liver plasma membranes, compared to the IgG-fraction of the preimmune serum. Then the IgG-fraction of the antiserum was preabsorbed with the membrane fatty acid binding protein or mGOT. Under these conditions the inhibition of membrane binding of [^{3}H]oleate was not affected by the mGOT preabsorbed antibody. In contrast, preabsorption of the IgG-fraction with the membrane fatty acid binding protein reversed the inhibitory effect on [^{3}H[oleate binding observed in presence of the antibody alone (Fig. 5).

These results underline the functional significance of the original membrane fatty acid binding protein and not that of mGOT in the membrane binding process of fatty acids.

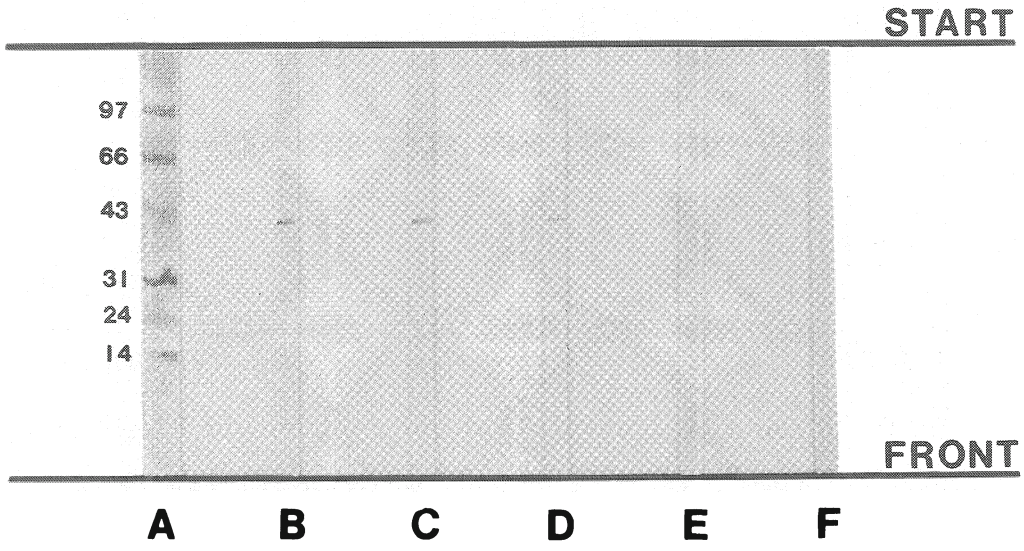


Fig. 4. Western blot analysis with the antiserum to the membrane fatty acid-binding protein and the authentic membrane fatty acid-binding protein (5 μg) (B), rat liver plasma membrane proteins (50 μg) (C), rat liver homogenate (200 μg) (D), the 100.000 g cytosolic supernatant (200 μg) (E), and rat mGOT (10 μg) (F). For controls, identical protein samples were incubated with the preimmune serum, detecting no staining with any sample (not shown). Coomassie blue stained molecular weight standards are illustrated in column A.

Discussion

In 1985 for the first time a membrane fatty acid binding protein with a molecular weight of 40 kD was described [8], which was clearly different from the previously described cytosolic fatty acid binding protein (molecular weight 14 kD). The initial isolation procedure of this protein included solubilization of the integral membrane proteins out of the phospholipid bilayer of purified rat liver plasma membranes by incubation with 1% (V/V) triton X100, removal of remaining membrane structures by centrifugation and extraction of excess of detergent down to about 0.1% triton X100 by passage over a Bio Beads SM-2 column. The solubilized membrane protein mixture was directly applied to an oleate-agarose affinity chromatography column. Proteins which were unspecifically retained to the hydrophobic matrix of the column were removed by extensive washing with high salt buffer. Proteins which were bound with high affinity to oleate-agarose were eluted with 8 M urea. This procedure is based on the functional property of the protein to bind fatty acids. It resulted in the isolation of a highly purified membrane fatty acid binding protein as judged by silver staining procedures (Fig. 1). After we raised a monospecific rabbit antibody to this protein [8], antibody inhibition studies became possible. In incubation experiments with isolated plasma membranes as well as with hepatocytes this antibody selectively inhibited membrane binding and cellular uptake of fatty acids, indicating the physiologic significance of this protein as a membrane fatty acid carrier [3–5, 8]. A similar protein was isolated by the same technique from rat heart and intestine [1, 7]. Recently the group of Berk *et al.* isolated a protein which appeared to be identical to the original membrane fatty acid binding protein using a different isolation method based on charge and size differences of molecules [15]. As a modification of the original procedure, they treated the plasma membranes with 2 M NaCl for solubilization of membrane proteins and applied the soluble NaCl protein extract to preparative isoelectric focusing. Proteins which migrated with pI's >9 were extracted and in a second step applied to HPLC gel permeation chromatography. All proteins with about 40 kD were

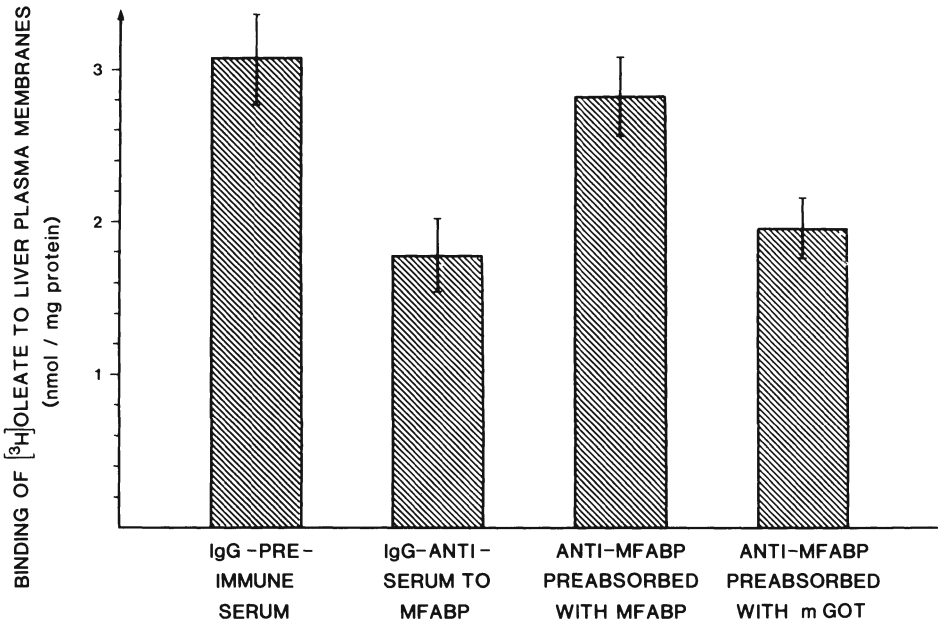


Fig. 5. Evaluation of the significance of the membrane fatty acid-binding protein (MFABP) and mGOT on binding of [^{3}H]oleate to isolated rat liver plasma membranes. In presence of the IgG-fraction of the antiserum to MFABP, binding was significantly reduced compared to controls pretreated with the IgG-fraction of the preimmune serum. Preabsorption of the IgG-fraction of the antiserum with MFABP reversed this inhibitory effect on [^{3}H]oleate membrane binding, whereas preabsorption with mGOT had no effect. Values are means ± S.D. of 3 replicate experiments.

then separated. Alternatively, oleate-agarose affinity chromatography was employed as second step after preparative isoelectric focusing [15, 16]. Such isolated proteins were then characterized. Amino acid sequence analysis of this protein revealed that the initial 24 amino acids were homologous to mGOT [16–18]. Further physiochemical characterization of the protein isolated by this procedure also confirmed the identity to mGOT [16–18]. Moreover, the protein showed GOT enzymatic activity [16–18]. From these results it was suggested that mGOT may participate in the translocation process of fatty acids across the plasma membrane. Since it would primarily not have been expected that this mitochondrial enzyme of amino acid metabolism plays any role in transport of fatty acids into cells, it was examined whether an antibody to mGOT has any effect on membrane transport of fatty acids. This antibody indeed inhibited in these experiments fatty acid uptake into isolated hepatocytes [16–18]. Therefore, it was suggested that cellular fatty acid uptake is mediated by a protein which shows complete N-terminal homology to mGOT and might even be identical to mGOT.

Based on this unexpected findings we tried to proof whether our membrane fatty acid protein, prepared by the originally described isolation procedure, is also homologous to mGOT. However, this could not be found in our studies. Direct comparison of the rat plasma membrane fatty acid binding protein and purified rat mGOT, provided by Drs. Passarella and Doonan, revealed that both proteins were not identical. There were small but distinct differences in molecular weight and IEP. On two-dimensional electrophoresis they migrated as two distinct spots (Fig. 2). Amino acid sequence analysis revealed that the membrane fatty acid binding protein is, in contrast to mGOT, blocked at the N-terminus and sequences of some fragments of this protein were not identical to mGOT [32]. Furthermore, ouchterlony immunodiffusion tests with the same monospecific polyclonal rabbit antibody to the membrane fatty acid binding protein,

which inhibited membrane binding and cellular uptake of fatty acids [3–8], showed precipitation with this membrane protein but not with mGOT (Fig. 3). This was supported by Western blot analysis using the same antibody (Fig. 4). Recent, yet unpublished data revealed that a cDNA probe of this membrane protein identified in Northern blots with intact rat liver RNA a mRNA signal of 1.6 kb, which is different to the 2.4 kb mRNA of rat mGOT. Most importantly, our membrane protein could bind [^{3}H]oleate, whereas mGOT showed no binding affinity for long chain fatty acids. All of these data confirm that our membrane fatty acid binding protein is distinct from the protein isolated by Berk *et al.* revealing homology to mGOT. The interpretation of this controversial finding could be, that both proteins are isolated by different procedures (see above). That GOT as abundant mitochondrial enzyme might also be a constituent of the plasma membrane seems unlikely, but cannot be excluded. Since it did not show binding affinity for fatty acids may indicate that it does not function as membrane fatty acid binding and translocation protein. However, surprising is the observation that antibodies to mGOT inhibited uptake of fatty acids in hepatocytes [16–18]. In this regards it would be of interest to evaluate the specificity of this antibody in a two-dimensional immunoblot and whether there is crossreactivity to our membrane fatty acid binding protein. It could also represent an unspecific interaction of this antibody at the plasma membrane level. If this would be the case, the meaning of antibody inhibition studies as indirect indicator for the membrane carrier function of a protein has to be questioned. Therefore, the function of putative carrier proteins has to be proven by direct evaluation of their transport capacity employing reconstitution experiments.

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Renal palmitate transport: possible sites for interaction with a plasma membrane fatty acid-binding protein

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Key words: kidney, renal basolateral membrane, renal brush border, albumin, membrane vesicles

Summary

In previous studies from this laboratory [14], a mediated transport system for long chain fatty acids was observed in rat renal basolateral membrane vesicles. Transport was measured in the absence of albumin and indicated the presence of a Na^+ independent anion exchange mechanism. The present experiments were done to characterize renal transport of fatty acids derived from fatty acid-albumin complexes. ^{3}H-palmitate uptake by brush border (BBMV) and basolateral membrane vesicles (BLMV) isolated from rat renal cortex was determined using a rapid filtration technique. All incubation media contained 100 μM ^{3}H-palmitate complexed to 100 μM bovine serum albumin. Up to 65% of initially bound fatty acid-albumin complexes were displaceable by washing with solution containing 0.1% albumin. Total palmitate uptake was measured as the remaining non-displaceable radioactivity. In BBMV in low ionic strength (300 mM mannitol) or ionic buffers (100 mM mannitol + 100 mM NaCl or KCl), total palmitate uptake at 15 sec did not differ from equilibrium (60 min) values of 10–11 nmoles/mg protein. Uptake was primarily due to binding. A similar pattern was seen with BLMV in 300 mM mannitol buffer. In BLMV in 100 mM NaCl or KCl buffers, equilibrium uptake was 10-fold lower than at 15 sec. This suggests binding followed by cation-dependent translocation. If a putative $FABP_{PM}$ is involved in transport only, its presence should be confined to BLMV.

Introduction

As in many other tissues, a large portion of the metabolic needs of the kidney are met by oxidative metabolism of non-esterified ('free') fatty acids. In considering regulation of fatty acid metabolism, it is generally accepted that plasma free fatty acid levels are a major determinant of long chain fatty acid entry into cells. There have been numerous studies attributing this process to simple diffusion in various tissues (e.g. 1–3). There is also considerable evidence for the presence of mediated transport systems for transmembrane fatty acid transport. For example, in adipocytes a mediated transport system has been demonstrated [4] which is subject to hormonal regulation [5, 6]. Mediated transport has also been described in heart [7, 8], intestine [9] and liver [10]. Isolation and characterization of a membrane fatty acid-binding protein ($FABP_{PM}$) from rat hepatocytes [11] spurred interest in the role of this class of protein in transmembrane fatty acid transport. Its potential involvement has been demonstrated by inhibition of fatty acid uptake by antibodies to $FABP_{PM}$ in hepatocytes [12] as well as in other tissues.

In the kidney, a number of studies have indicated an interaction of long chain fatty acids with the transport of organic anions [13]. Recently, experiments from this laboratory have demonstrated an anion exchange system for long chain fatty acids in rat renal basolateral membrane vesicles [14]. The latter experiments were done in the absence of

albumin in contrast to all other studies of fatty acid transport. The present experiments were thus carried out to characterize the effect of albumin on palmitate transport by brush border (BBMV) and basolateral membrane vesicles (BLMV) isolated from rat renal cortex. Under all conditions tested, total palmitate uptake by BBMV could be accounted for by binding rather than translocation. In BLMV, apparent fatty acid translocation was cation (Na^+ or K^+) dependent. If a putative $FABP_{PM}$ were involved solely in transport, its location should thus be confined to the BLMV.

Experimental procedure

Brush border (BBMV) and basolateral membrane vesicles (BLMV) were prepared from kidney cortical tissue obtained from male Sprague Dawley rats weighing 180–400 g. The method for vesicle isolation and identification was similar to that described previously [14, 15] in which membranes were subjected to differential centrifugation followed by precipitation of all but BBMV in a divalent cation buffer. BLMV were further purified on a discontinuous sucrose gradient (8%/38.7%, w/w).

A rapid filtration technique was used to measure uptake of [9,10-^{3}H]palmitate (NEN, 28.5–30 Ci/mmol) by isolated vesicles. At zero time, 15–25 μl of vesicle suspension containing 8–10 mg protein/ml were added to 165 μl of incubation mixture. All experiments were carried out at 20–23° C. At various time intervals ^{3}H-palmitate uptake was terminated by addition of 2 ml ice-cold 'stop' solution followed by rapid filtration through 0.45 μm Millipore nitrocellulose filters. Filters were washed twice more with 'stop' (stop/wash) solution and were assayed for radioactivity by liquid scintillation counting. In all experiments, corrections were made for binding of ^{3}H-palmitate to filters by running appropriate filter controls containing incubation solution minus vesicles. Duplicate determinations were made for each experimental time period and all experiments were carried out in at least 3 different membrane preparations.

Vesicles were prepared in solution containing 300 mM mannitol and 20 mM Tris-HEPES, pH 7.4. The incubation media were isoosmotic to the vesicle suspension solution. All incubation media contained 100 μM ^{3}H-palmitate complexed to 100 μM bovine serum albumin (Sigma, Fraction V) in addition to 20 mM Tris-HEPES, pH 7.4 and either 300 mM mannitol or 100 mM mannitol plus 100 mM NaCl or KCl. The stop/wash solutions were matched to the appropriate incubation solutions with respect to mannitol, NaCl and KCl concentrations. In addition, the stop/wash solutions contained 5 mM Tris-HEPES, pH 7.4, 1 mM probenecid and, except where noted (see Fig. 1), 0.1% albumin. Probenecid was added to decrease binding of ^{3}H-palmitate to filters.

Binding of ^{3}H-palmitate to vesicles was measured by the osmotic method as previously described [14]. Briefly, osmolality of the incubation medium was increased by addition of sucrose. Increased osmolality causes a decrease in intravesicular volume. Extrapolation of the data to uptake at zero intravesicular volume permits an estimate of binding.

Results

When albumin was present in the incubation medium, total palmitate uptake by isolated BBMV reached a peak by 30 sec (Fig. 1) and leveled off thereafter. When albumin was also included in the stop/wash solution, total uptake was reduced by approximately two-thirds. It thus appears that a large portion of the fatty acid-albumin complexes were loosely bound to external membrane sites in such a manner as to be easily displaceable by albumin. Increasing albumin concentration of the stop/wash solution did not further increase the amount of radioactivity displaced (data not shown). Approximately 10 nmoles/mg protein were associated with BBMV in non-readily reversible fashion (Fig. 1). This represents a high degree of binding. In comparison, D-glucose is a solute which does not bind extensively to membranes. In BBMV the volume of distribution of D-glucose at equilibrium is approximately 2–3 μl/mg protein. If 100 mM palmitate did not bind to protein and equilibrated with the intravesicular space, the calculated volume of

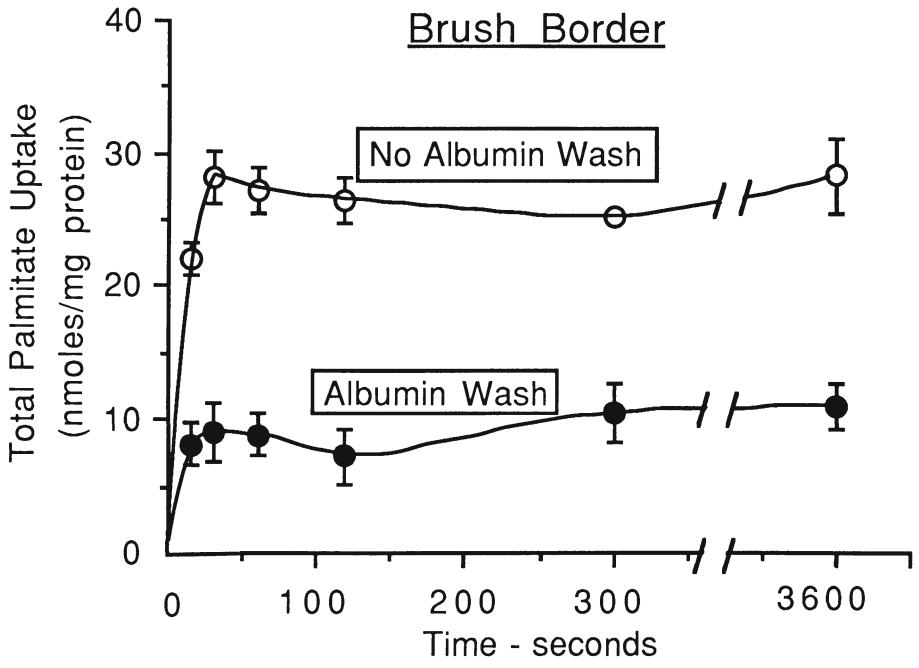

Fig. 1. Comparison of total palmitate uptake by brush border membrane vesicles in the presence and absence of albumin in the stop/wash solution. Incubation mixture contained 300 mM mannitol, 20 mM Tris-HEPES, pH 7.4 with 100 μM ^{3}H-palmitate and 100 μM albumin. The stop/wash solution (see *Experimental Procedures*) was used with or without 0.1% albumin. N = 4 membrane preparations. Values are mean ± SE.

distribution (uptake/mg protein ÷ medium concentration) would be close to 100 μl/mg protein. Since actual vesicle size does not change to that extent, a large calculated volume of distribution is indicative of substantial binding. Extensive binding of ^{3}H-palmitate to BBMV was confirmed in experiments using the osmotic method. After 20 min incubation in 300 mM mannitol buffer, binding accounted for 90 ± 13% of total uptake (N = 5 membrane preparations).

Since Na^+ has been implicated in possible mediated transport of long chain fatty acids [10, 16], the effects of inwardly directed cation concentration gradients on palmitate uptake were examined. Figure 2 shows that, in BBMV, ionic composition of the incubation medium had no significant effect on initial or equilibrium ^{3}H-palmitate uptake. In contrast, ^{3}H-palmitate uptake by BLMV was markedly affected by medium composition (Fig. 3). Initial total uptake was highest in mannitol buffer and equilibrium values were not significantly different from initial uptake values. In the presence of extravesicular Na^+, total ^{3}H-palmitate uptake at equilibrium was at least 10-fold lower than initial uptake. This effect was not specific for Na^+ as it was also observed in the presence of extravesicular K^+. Measurement of total uptake does not distinguish between binding and transport into intravesicular space. However, the time dependent decrease in total uptake could be accounted for by binding of fatty acid-albumin complexes followed by translocation across the membrane and subsequent efflux.

Discussion

Fatty acid-albumin complexes bind rapidly to renal

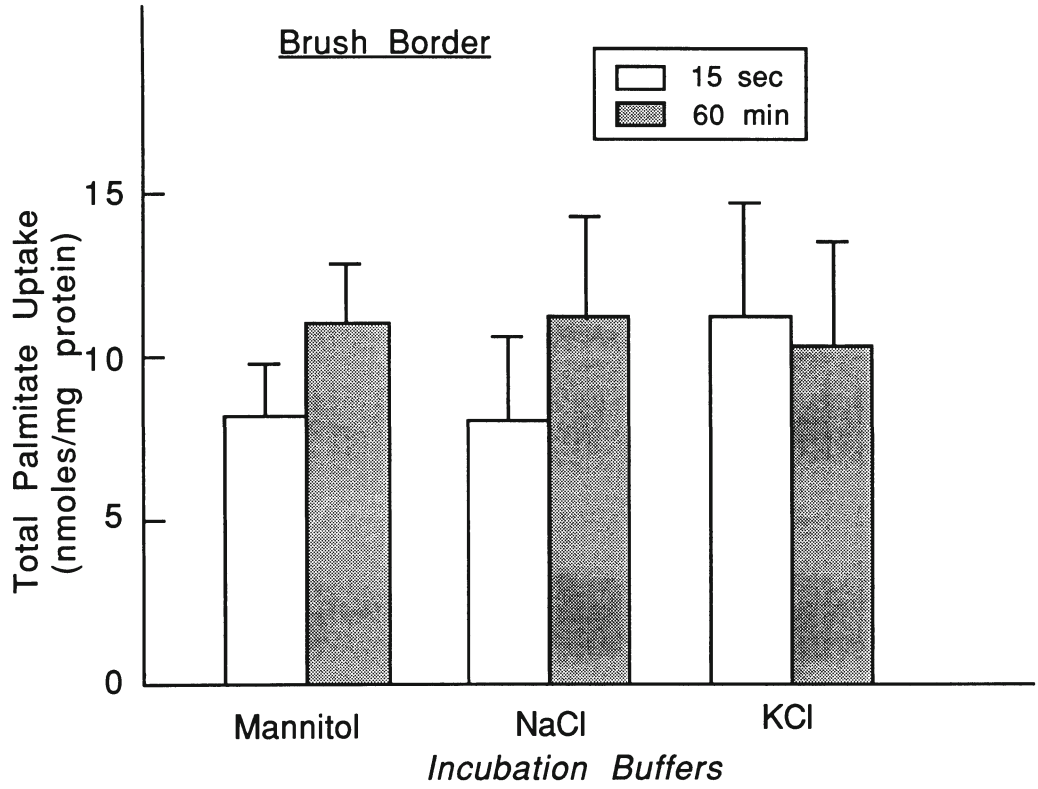

Fig. 2. Effect of various incubation buffers on total palmitate uptake by brush border membrane vesicles. Vesicles were prepared in 300 mM mannitol, 20 mM Tris-HEPES, pH 7.4. Incubation mixtures contained 20 mM Tris-HEPES, pH 7.4 and 300 mM mannitol (N = 4), 100 mM mannitol plus 100 mM NaCl (N = 8) or KCL (N = 3) in addition to 100 μM ^{3}H-palmitate and 100 μM albumin. The stop/wash solutions contained 0.1% albumin.

plasma membranes. A portion of the membrane associated complexes can be displaced by washing with albumin-containing solutions. The remaining radioactivity is not readily dissociable from membranes. It is not yet clear whether the membrane associated ^{3}H-palmitate remains complexed to albumin. The patterns of ^{3}H-palmitate association with BBMV and BLMV show distinct differences. In BBMV, under all conditions tested, and in BLMV in low ionic strength buffer (300 mM mannitol), ^{3}H-palmitate derived from fatty acid-albumin complexes bound rapidly and remained bound for up to 60 min. However, when NaCl or KCl was present in the extravesicular solution ^{3}H-palmitate associated with BLMV decreased with time. One possible interpretation of these results would be that, following binding of a fatty acid-albumin complex to BLMV, whether or not translocation fatty acid into the vesicle interior (influx) occurs depends upon the presence of extravesicular cations. It is unknown whether such translocation is a mediated process or simple diffusion. The decrease in ^{3}H-palmitate association with BLMV with time could reflect efflux of intravesicular free fatty acid which would be rebound to albumin in the stop/wash solution.

In previous experiments from this laboratory, we described a mediated transport system for palmitate in BLMV [14]. In contrast to the present work, the former experiments were carried out in the absence of albumin. Under such conditions, mediated palmitate transport occurred via an anion ex-

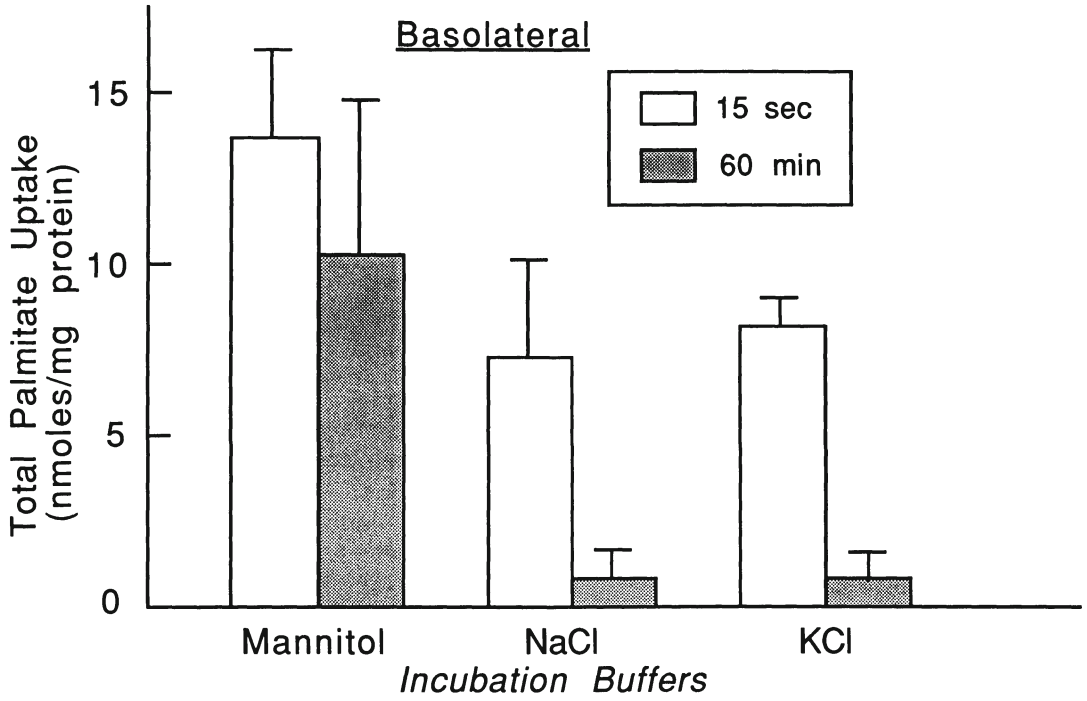

Fig. 3. Effect of various incubation buffers on total palmitate uptake by basolateral membrane vesicles. Conditions were the same as in Fig. 2. Mannitol (N = 3); NaCl (N = 4); KCl (N = 3).

change mechanism which showed interaction with the transport system for the organic anion, para-aminohippurate (PAH). Additionally, palmitate transport was unaffected by the presence of an inwardly directed Na^+ concentration gradient. In the present studies with albumin present, Na^+ did have an effect on interaction of ^{3}H-palmitate with the BLMV. Since the major difference between the two studies was the presence or absence of albumin in the incubation mixture, it is tempting to speculate that univalent cations may, in some fashion, affect interaction of the palmitate-albumin complexes with BLMV. Recent studies have provided evidence that adsorption to hepatocyte membranes may induce conformational changes in the albumin molecule [17, 18]. However, nothing is known of possible effects of such changes in conformation or ionic environment on release of ligand.

The effect of Na^+ on fatty acid transport in the presence of albumin in other systems has produced conflicting results. In recent experiments in the isolated perfused liver, cell membrane potential difference and fatty acid uptake were monitored continuously during ion substitution experiments. Results strongly support the existence of electrogenic Na^+ cotransport [16]. In BLMV isolated from rat hepatocytes, imposition of an inwardly directed Na^+ concentration gradient resulted in stimulation of ^{3}H-oleate uptake. Stimulation of uptake was not observed in the presence of a similar K^+ concentration gradient [10]. These findings are also consistent with a Na^+ co-transport system. In contrast, as mentioned above, Na^+ was without effect on ^{3}H-palmitate transport by rat renal BL MV in the absence of albumin. In the presence of albumin, while an inwardly directed Na^+ concentration gradient appeared to result in fatty acid translocation, the same effect was observed with an inwardly directed K^+ gradient (Fig. 3) and with equal intra- and extravesicular sodium concentrations (data not shown). Differences observed between liver and kidney could lie in differences in

experimental technique or may reflect real tissue differences.

In recent years, considerable interest has focused on the role of a plasma membrane fatty acid binding protein ($FABP_{PM}$) in the process of fatty acid transport into a number of cell types. Originally described in membranes isolated from rat hepatocytes [11], similar 40 kDa proteins have since been observed in intestine [9], heart [7, 8], and adipose tissue [19]. In the kidney, a fatty acid binding protein derived from rat basolateral membranes has been described [20]. However, the specificity of this 56 kDa protein for other organic anions was not examined. The results of the present study would allow certain predictions with respect to the possible site and role of a $FABP_{PM}$ in the kidney. First, if a $FABP_{PM}$ were present in BBMV, its role would be related to binding or possible incorporation of fatty acids into membrane lipids rather than fatty acid translocation. Secondly, in BLMV an antibody to a putative $FABP_{PM}$ might block the time dependent decrease in ^{3}H-palmitate uptake observed in the presence of extravesicular cations. If the $FABP_{PM}$ were only involved in transport, it would be expected that other transport systems for palmitate such as an anion exchanger or simple diffusion would still be expressed. An important issue which remains unresolved is whether the effect of cations on renal palmitate transport reflects interactions with the albumin molecule or with a membrane protein.

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Fatty acid-binding to erythrocyte ghost membranes and transmembrane movement

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Key words: palmitate-binding, erythrocyte ghost, membrane transport, dissociation rate constants, efflux kinetics, $FABP_{PM}$

Summary

Palmitate binding to human erythrocyte ghost membranes has been investigated with ghost preparations suspended in 0.2% albumin solutions. Free unbound palmitate in the extracellular water phase was measured in equilibrium studies using albumin-filled acid loaded ghosts as small semipermeable bags. The apparent dissociation constant of binding to the membrane is 13.5 nM and the binding capacity 19 nmoles per 7.2×10^9 cells.

The 0° C exchange efflux kinetics of palmitate from albumin-filled ghosts is described by a model, which provides estimates of the rate constant of membrane transfer, $k_3 = 0.024\,s^{-1}$, independent of the molar ratio of palmitate to albumin (ν) and of a mean dissociation rate constant of the palmitate-albumin complex, $k_1 = 0.0015\,s^{-1}$ at ν 0.2, allowing for a heterogeneity of the palmitate binding to albumin.

The values of a third kinetically determined ν dependent model constant, Q, the ratio of palmitate bound to the membrane inner surface to palmitate on intracellular albumin, are not different from the Q values obtained by equilibrium experiments.

The temperature dependences of k_1 and k_3 in the interval 0° C to 15° C give activation energies of 96 and 103 kJ/mole, respectively. The 0° C exchange efflux increases about 2 fold in response to a rise of pH from 6 to 9. The results suggest a carrier mediated palmitate flux at low ν with a V_{max} about 2 pmoles $min^{-1}\,cm^{-2}$ at 0° C pH 7.3.

Introduction

The generally accepted idea that cell membrane transfer of long-chain fatty acids is governed by phase partition between water and the lipid bilayer has in recent years been challenged. Investigations of fatty acid uptake by adipocytes [1, 2], cardiac myocytes [3, 4] and hepatocytes [5] indicate that a plasma membrane fatty acid binding protein ($FABP_{PM}$) is crucial for the uptake. To gain insight into the general mechanism by which fatty acids permeate cellular membranes, we turned our attention to erythrocytes. Isolated cell membranes of erythrocytes (ghosts) are particularly suitable for such investigations for several reasons. Firstly no fatty acid metabolism occur and fatty acid uptake on the membrane from albumin solutions is readily measured. Secondly the water phase concentration extracellularly can be determined in equilibrium with fatty acid bound to albumin intracellularly. Thirdly efflux kinetics is easily recorded of fatty acids from such ghosts. Potentially important is that the rate of release from albumin is optional in the choice of the albumin concentration.

In the present work with palmitate we have observed a rapid initial efflux of a tracer fraction, which is much greater than the rate of release from the albumin pool. A suggested membrane uptake of palmitate from albumin solutions was confirmed by equilibrium studies and the corresponding model of efflux kinetics was worked out.

The validity of our model appears from the agreement of the membrane binding estimated kinetically with that measured directly by equilibrium studies at various molar ratio of palmitate to albumin (ν). Moreover values of the estimated rate constant of dissociation of palmitate – albumin complex are in the expected range. Together with results from temperature and pH effects on the rate constants and the fluxes, the main conclusion is that a membrane component is responsible for the binding and for the transmembrane movement of palmitic acid across the erythrocyte membrane.

Experimental procedures

Ghosts were prepared by the method of Schwoch and Passow [6], carried out as described by Funder and Wieth [7]. Preparation of ghosts with 0.2% intracellular bovine serum albumin (BSA) was carried out by adding BSA to the haemolysing solution. [^{125}I] BSA was used to control the concentration of BSA in isolated washed ghosts and to verify that no leakage of BSA occurs during the efflux experiments. The ghosts were stored in 165 mM KCl, 2 mM phosphate buffer, pH 7.3, containing 0.02 mM EDTA/EGTA (1 : 1) with or without 0.2% BSA for up to 48 hours. [(9,10)-^{3}H] Palmitic acid media containing 0.2% BSA (charge buffers) for labelling of ghosts were prepared as described by Bojesen [8]. BSA-filled ghosts were gently packed by 5 min centrifugation at 23 500 × g at 0° C and labelled by equilibrating the ghosts with charge buffer (1 : 1.5 v/v) either for 50 min at 0° C, for 20 min at 5° C, for 10 min at 10° C or for 5 min at 15° C. This procedure ascertains that the state of the membrane remains the same during the preparation and the experiments.

Equilibrium experiments

The uptake of palmitate on ghosts at 0° C was measured by equilibrating 500 mg packed BSA-free ghosts with 750 μl charge buffer of different ν from 0.05 to 1.5.

The free water phase concentration of palmitate in equilibrium with palmitate complexed to 0.2% BSA at various ν was determined by 0.2% BSA-filled labelled ghosts as small semipermeable bags. Such ghosts were washed with 3 × 10 volumen KCl-phosphate buffer to remove the charge buffer and left for 50 min at 0° C. After centrifugation, 15 min at 50 000 × g, aliquots were taken of the water phase for counting.

Efflux experiments

Labelled ghosts were washed and packed in plastic tubes at 0° C, (5° C, 10° C or 15° C, respectively) for 15 min at 50 000 × g. The supernatant was removed by cutting the tubes just below the interface and 100–140 μl packed ghosts were injected into 30–40 ml stirred isotope-free 165 mM KCl, 2 mM phosphate medium at appropriate temperature and pH containing 0.2% BSA and inactive palmitate with ν equal to the intracellular ν. Phosphate buffer at pH 9 was obtained by titration with 1 M TRISMA-base in 165 mM KCl. Using the Millipore-Swinnex filtration technique [9], ten to fifteen samples (including an equilibrium sample) were taken at appropriate time intervals for determination of the extracellular accumulation of ^{3}H palmitate as a function of time. The sampling time corresponds to about 70–80% equilibrium, which ascertains negligeable backflow of tracer, less than 1%.

Efflux kinetics

^{3}H palmitate efflux from ghosts is described by an efflux model based upon the existence of a transport pool of ^{3}H palmitate bound to the internal surface of ghost membrane (b_i) and a corresponding external surface pool (b_y) (Fig. 1). Furthermore the dissociation rate constants (k_5 and k_5^*) of palmi-

tate from the membrane pools b_i and b_y are both assumed to be higher than the rate constants (k_1 and k_3) of dissociation of the palmitate-albumin complex and of transfer through the membrane, respectively. An assumption which turns out to be correct in the case of k_5^* (see Results).

Equations expressing the rates at which the different pools of ^{3}H palmitate (Fig. 1) are receiving and loosing palmitate result in a second-order differential equation with constant coefficients

$$\frac{dY^2}{dt^2} + (k_3 + k_1 + k_1/Q)\frac{dY}{dt} + k_1 k_3 Y = k_1 k_3 T/W$$

where Q is the ratio of palmitate bound on the internal surface of ghost membrane to palmitate bound to albumin (b_i/a, see Fig. 1), W the extracellular volume and T the total amount of ^{3}H palmitate. This equation has the solution

$$1 - Y/Y_\infty = -CI\ W/T\ e^{-\alpha t} - C2\ W/T\ e^{-\beta t} \qquad Y_\infty = T/W\ (t = \infty)$$

CI and C2 being the integration constants. The rate constants (k_1 and k_3) and Q are calculated from the three equations:

$$k_1 = \alpha \cdot \beta / k_3 \quad (I)$$

$$k_3 = \alpha + \beta + \frac{\alpha \cdot \beta \cdot T}{W(\alpha CI + \beta C2)} \quad (II)$$

$$Q = \frac{b_i}{a} = \frac{1}{k_3 T/(-\alpha CI - \beta C2)W - 1} \quad (III)$$

A presentation of the mathematics of model equations will be published elsewhere.

Results

Equilibrium experiments

An apparent K_d of membrane binding of palmitate is

$$K_d = \frac{F(T_c - B)}{B}$$

where F is the concentration of palmitate in the waterphase, T_c the total amount of binding capacity and B the membrane bound palmitate.

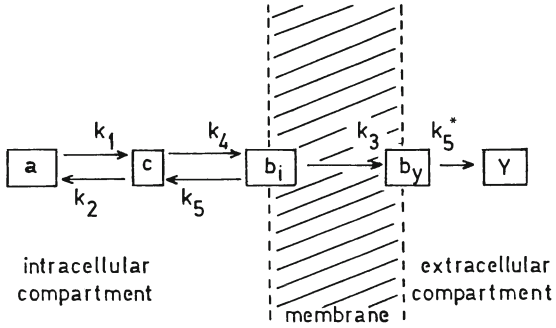

Fig. 1. The compartmental model which accounts for the efflux kinetics of albumin bound tracer palmitate in ghosts. Arrows and rate constants indicate the flow of tracer palmitate between compartments. a is the intracellular albumin-tracer palmitate complex, c is the intracellular free unbound tracer palmitate, b_i is the tracer palmitate bound to the internal surface of ghost membrane, b_y is the tracer palmitate bound to the external surface of ghost membrane, all per ml cell water, Y is the extracellular tracer palmitate per ml extracellular medium, k_1, k_2, k_3, k_4, k_5 and k_5^* are rate constants.

F and B are determined by the described equilibrium experiments. Rearranging this equation gives

$$1/F = (T_c/K_d)\ 1/B - 1/K_d$$

Thus K_d and T_c can be determined by a double reciprocal plot. From linear regression (correlation coefficient $r = 0.96$ ($n = 19$)) K_d is estimated to 13.5 ± 5 nM and T_c to 19.4 ± 7.3 nmoles per g packed ghosts (7.2×10^9 cells) at 0° C, pH 7.3.

Figure 2 shows the efflux kinetics of ^{3}H palmitate from BSA-filled ghosts into the surrounding medium. The experimental curve can apparently be represented by the sum of two exponentials. Curve-fitting by the curve peeling method and by the weighted least squares procedure give indistinguishable results. However, scrutinizing the curves revealed that the model is not quite correct since the slope of the slow phase was always decreasing in contrast to a model based computer simulation.

Three domains of long chain fatty acid binding to BSA are described by Brown & Shockley [10], substantiating the suggested three high affinity binding sites [11]. The following analytical procedure is therefore based upon the assumption that

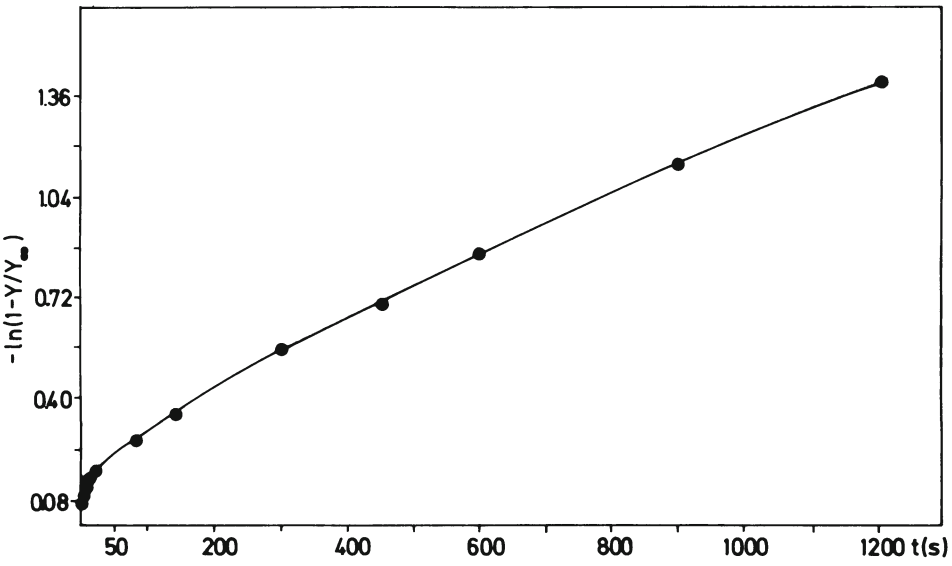

Fig. 2. Exchange efflux kinetics of tracer palmitate from ghosts loaded with 0.2% albumin into the extracellular medium at 0° C, pH 7.3, $\nu = 0.2$, a reversed semilogarithmic plot. Y is the tracer acid in the extracellular medium at any time of sampling and Y_∞ the equilibrium value.

the decreasing slope of the slow phase is caused only by a decreasing effective k_1 and that the about 1 : 2 : 4 proportion of equilibrium constants of three high affinity binding sites [11] reflects the proportion of three k_1 components. Using the initial section of the slow phase we obtain by curve peeling and by application of equations (I), (II) and (III) the initial integrated mean of k_1, the corresponding k_3 and Q values. At 0° C, pH 7.3 and $\nu = 0.2$ we get $k_1 = 1.47 \pm 0.03 \times 10^{-3}\ s^{-1}$, $k_3 = 24.0 \pm 1.8 \times 10^{-3}\ s^{-1}$ and $Q = 0.20 \pm 0.01$ ($n = 9$). These values for k_3, Q and 0.9, 1.8 and $3.6 \times 10^{-3}\ s^{-1}$ for the k_1 components account according to computer simulation precisely for the experimental curves. The mathematics of the computer simulation is published elsewhere.

With higher ν, k_3 is not different whereas k_1 increases as expected. Thus at $\nu = 0.6$, k_3 is $26.8 \pm 1.9 \times 10^{-3}\ s^{-1}$ and the initial integrated mean k_1 is $2.56 \pm 0.08 \times 10^{-3}\ s^{-1}$ ($n = 9$). The three k_1 components fitting computer simulation are 1.4, 2.8 and $5.6 \times 10^{-3}\ s^{-1}$.

The intercept with the ordinate (Fig. 2) represents an instantaneously released tracer fraction of 6–8%, the 'blank'. The initial leak of BSA is less than 1% and an extracellularly trapped volume of charge buffer in packed ghosts has been excluded. Therefore this fraction indicates a tracer fraction bound to the outer surface of the membrane. This corroborates our contention that the rate constant of dissolution from the membrane binding (k_5^*) is much higher than k_3 and k_1.

The equilibrium experiments enable us to determine a Q value, which includes a pool of palmitate located at the outer surface. The validity of our model is strongly supported by the fact that this Q value, corrected for this pool does not differ significantly from the kinetically determined Q defined by equation (III).

In order to determine k_3 without interference of k_1, efflux experiments are performed with BSA-free ghosts loaded with palmitate at 0° C, pH 7.3, $\nu = 0.2$. These experiments show a monoexponential efflux curve with a k_3 of $30.0 \pm 0.3 \times 10^{-3}\ s^{-1}$ ($n = 4$) and a 'blank' of 6–8%. These results strongly support our model with a fast release of about 6–8% of palmitate located on the external surface of the membrane and a transfer of palmitate from the internal located pool with the rate constant k_3.

Temperature effects

The temperature dependences of k_3 and of the initial mean k_1 values were investigated in the interval between 0° C and 15° C. Beyond 15° C the time resolution of our technique is poor. Figure 3 shows the Arrhenius diagrams. The relations are linear and the activation energies are 103.0 ± 4.5 kJmole^{-1} and 96.1 ± 2.2 kJmole^{-1} for k_3 and k_1, respectively.

Effects of pH

The initial exchange efflux of palmitate is calculated independently of the interpretation of the kinetics. At 0° C and $\nu = 0.2$ the values for pH 6.0, 7.3 and 9.0 are 84.0 ± 8.4 × 10^{-3}, 99.0 ± 6.6 × 10^{-3} and 198.0 ± 16.8 × 10^{-3} pmoles min^{-1} cm^{-2}, $n = 5$ in all cases. At 0° C and $\nu = 0.6$ the corresponding values are 300 ± 20 × 10^{-3}, 635 ± 48 × 10^{-3} and 702 ± 72 × 10^{-3} pmoles min^{-1} cm^{-2}, $n = 5$ in all cases. Thus the initial exchange efflux increases more than two fold, when pH is increased from 6.0 to 9.0 ($P < 0.001$). This flux dependence of pH is caused by a change of k_3 whereas Q is not significantly pH dependent. The efflux dependence of ν is caused only by the ν dependent membrane binding.

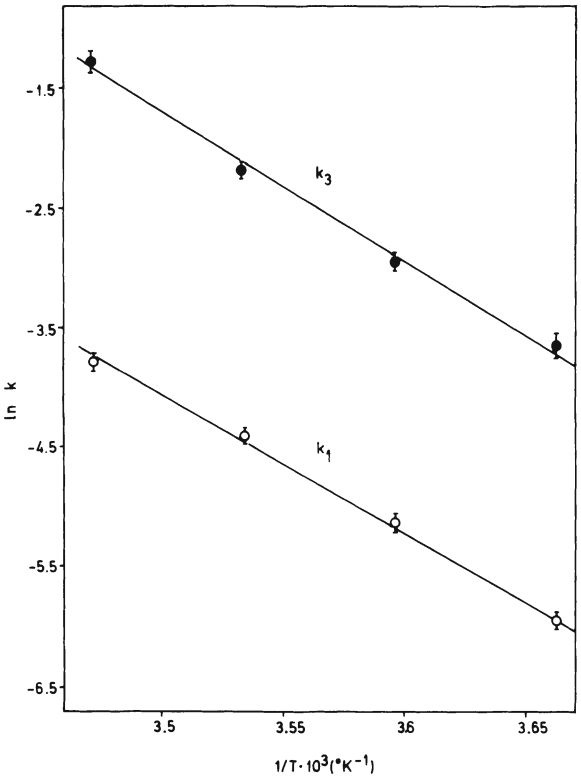

Fig. 3. Arrhenius plot of the initial mean dissociation rate constant of palmitate-albumin complex (k_1) (○) and of the rate constant of transfer through the membrane (k_3) (●). $\nu = 0.6$, pH = 7.3.

Discussion

By means of albumin-filled ghost preparations it has been possible to investigate the rate constant of palmitate dissociation from BSA and the rate constant of palmitate transfer across the ghost membrane. In addition the investigations have provided further information as to the heterogeneity of the palmitate binding to BSA. This was revealed by the continuous decrease of the slope of the efflux curve and by the complete fit of a computer simulated curve assuming three k_1 components in the proportion 1 : 2 : 4. However, we cannot conclude that this reflects prevailing equilibrium constants of three constitutive BSA binding sites, since the three k_1 values, fitting the data, are higher at $\nu = 0.6$ than at $\nu = 0.2$. Until more information is available our interpretation of the heterogeneity of k_1 remains formal. No previous measurements of rate constants have demonstrated the expected heterogeneity but we may assume that our initial mean k_1 is comparable to the rate constants measured by others. Allowing for different temperatures, albumin types, fatty acids and ν values the discrepancies are quite small. Thus Scheider [12] found dissociation rate constants in the range 2.6 to 3.7 × 10^{-3} s^{-1} of 0° C for oleate, linoleate and laurate bound to human and/or bovine serum albumin with a ν value not greater than 1. Svenson *et al.* [13] found 42 × 10^{-3} s^{-1} for the dissociation rate constant of palmitate-human serum albumin complex at 25° C ($\nu = 0.95$) which is similar to our initial

mean k_1 at 15° C ($\nu = 0.6$) of $23.3 \pm 0.8 \times 10^{-3}$ s^{-1} allowing for different temperatures.

The observed activation energy of our k_1 is about 30 kJ/mole higher than the activation energy reported by Svenson *et al.* [13] between 9° C and 25° C, but similar to the value, 80 ± 12.5 kJ/mole for laurate reported by Scheider between 0° C and 21° C.

Our efflux experiments with BSA-free ghosts conclusively demonstrate that the rate constant k_3 is independent of the presence of BSA and therefore can be ascribed to the transmembrane movement of palmitate. It is independent of ν and has not previously been measured.

An approximate maximum flux (J_{max}) of palmitate across the ghost membrane, 2 pmoles min^{-1} cm^{-2}, can be calculated from the rate constant and the maximum binding capacity as $J_{max} = k_3 \times T_c/A$ neglecting the 6–8% palmitate bound to the external surface of ghost membrane. A is the area of ghost membrane. Recalculations of the maximum uptake of palmitate and/or oleate by adipocytes [1, 2], cardiac myocytes [3, 4] and hepatocytes [5] is in the range from 25 to 300 pmoles min^{-1} cm^{-2} at 23° C/37° C. The lower temperature taken in consideration, the ghost membrane is nearly as competent to transport fatty acids. This is quite unexpected since the erythrocytes are not a cell-type specialized for uptake and utilization of fatty acids like cardiac myocytes, adipocytes and hepatocytes. Our experiments do not point to a transport of protonated palmitate. The activation energy of palmitate transfer through the erythrocyte membrane is much higher than that of salicylate [9] which is believed to permeate the membrane through the lipid bilayer as protonated acid. Moreover the palmitate efflux rate from ghosts increases with increasing pH because the rate constant of transfer increases whereas the efflux rate of salicylate decreases tenfold by increasing pH from 6 to 7.3. Our investigations speak against an adsorption of palmitate into phospholipid bilayer and a translocation by a simple diffusion through the bilayer. Firstly studies on phospholipid bilayers [14] show no limited capacity of fatty acid binding. Up to 2 moles of fatty acids per mole phospholipid can be incorporated into bilayers, whereas our study indicates a maximum of 1 mole palmitate per about 70 moles phospholipids (19 nmoles per 7.2×10^9 cells). Secondly the determined rate constants for fatty acid translocation through phospholipid bilayers [15, 16] are two to three orders of magnitude lower than the ν independent k_3 of the present study, our low temperature taken in consideration. Therefore we conclude that a single membrane component (probably a protein) is responsible for the binding and for the translocation of palmitate across the human erythrocyte ghost membrane.

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Acyl-CoA-binding protein (ACBP) and its relation to fatty acid-binding protein (FABP): an overview

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Key words: fatty acid-binding proteins, acyl CoA-binding proteins

Summary

Acyl-CoA-binding protein is a 10 Kd protein which binds medium- and long-chain acyl-CoA esters with high affinity. The concentration in liver is 2–4 times the acyl-CoA concentration. ACBP has much greater affinity for acyl-CoA than FABP. FABP from bovine heart and liver is unable to compete with multilamellar liposomes, Lipidex and microsomal membrane in binding acyl-CoA esters, whereas ACBP effectively extracts acyl-CoA from all those sources. Previously published results on the effect of FABP on acyl-CoA metabolism need to be reevaluated due to possible contamination with ACBP. Recently it was discovered that ACBP is identical to a putative neurotransmitter diazepam binding inhibitor. The possibility therefore exists that ACBP has more than one function.

Discovery of acyl-CoA-binding protein and its identity with diazepam binding inhibitor protein

Termination of fatty acid synthesis at medium-chain length (C_8–C_{12}) by goat mammary gland fatty acid synthetase is mediated by a medium-chain acyltransferase and the resulting products are acyl-CoA esters [1]. The acyltransferase involved is an inherent part of the synthetase complex and identical to the loading transferase [2, 3].

In order to be active in termination of fatty acid synthesis at medium-chain length the goat fatty acid synthetase requires the presence of an acyl-CoA utilising system in form of an acyl-CoA-binding molecule or acyl-CoA utilising enzyme system. Bovine serum albumin, methylated α-cyclodextrin and the microsomal triacylglycerol synthesising system are all able to fulfil this function [1, 4, 5].

In vivo fatty acid synthetase is believed to be a cytosolic enzyme and the triacylglycerol synthesising enzymes are microsomal bound. An intracellular transport molecule is therefore required to mediate the transport of acyl-CoA esters from the fatty acid synthetase to the microsomal triacylglycerol synthesising enzymes in goat mammary gland. Fatty acid-binding protein (FABP) has by a number of groups been suggested to be involved in intracellular storage and transport of acyl-CoA esters [6].

We therefore tested FABP to see if it had the ability to induce medium-chain fatty acid synthesis by goat mammary gland fatty acid synthetase. The result was rather surprising because pure bovine liver FABP was unable to induce medium-chain length fatty acid synthesis [7]. However, partly purified FABP contained a novel 10 Kd protein which could induce medium-chain fatty acid synthesis by goat mammary fatty acid synthetase [7]. This protein was named acyl-CoA-binding protein (AC BP).

Recently, it was discovered that ACBP has a sequence identical to that of a protein called diazepam binding inhibitor (DBI) protein [8]. The latter protein was originally isolated from mammalian

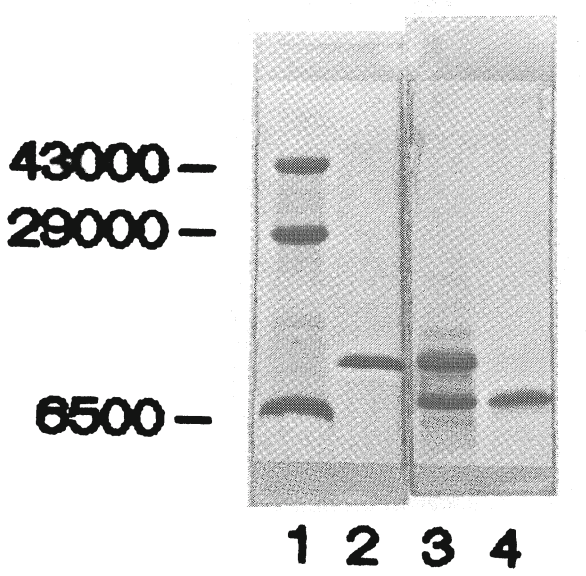

Fig. 1. Polyacrylamide electrophoresis of molecular weight markers (lane 1). (aprotenin 6.500, carboanhydrase 29.000 and ovalbumin 43.000), bovine liver FABP (lane 2). G-50 protein (lane 3) and bovine ACBP (lane 4). Electrophoresis was carried out on a pharmacia phast system using 20% homogeneous gels as recommended by the manufacturer.

brain, based on its ability to displace diazepam and other benzodiazepines from the benzodiazepine binding site on the γ-aminobutyric acid ($GABA_A$) receptor complex [9, 10]. It has been suggested that DBI is the natural neurotransmitter which acts through the benzodiazepine binding site on the $GABA_A$ receptor complex [9].

For the moment it is not clear if ACBP, besides being an acyl-CoA binding protein, is a neurotransmitter too. There are major evidences which question the role of ACBP (DBI) as neurotransmitter. The most important are: 1. The diazepam binding inhibitory activity of ACBP (DBI) is rather low, μmolar concentrations are needed [11]; 2. ACBP (DBI) is found in all tissues tested. Furthermore the concentration found in peripheral tissue is in some cases higher than in brain, i.e. liver contains 3 times more ACBP than brain [12]; 3. Direct binding of ACBP (DBI) to the $GABA_A$ receptor has not been shown; 4. Using highly purified ACBP from bovine and rat liver we have been unable to repeat that ACBP (DBI) has diazepam binding inhibitory activity [14]; 5. cDNA clones of DBI do not contain a leader signal sequence as it would be expected of a secreted protein [15].

We therefore think that ACBP is an acyl-CoA carrier and storage protein rather than a neurotransmitter.

Purification on molecular properties

The method used to purify ACBP is a further development of the procedure described by Haunerland *et al.* [16] for purification of bovine liver FABP. The method includes the following steps: 1. acid precipitation (pH 4.0), 2, heat treatment (50° C), 3. gelfiltration chromatography on Sephacryl S-200, 4. gelfiltration on Biogel P-10 and 5. isoelectric focusing. We have replaced the Sephacryl S-200 and the Biogel P-10 column with Sephadex G-100 and Sephadex G-50, respectively. The isoelectric focusing step is replaced by a reverse phase h.p.l.c. step [17]. Using this procedure, we obtained a protein which gives a single band on gelelectrophoresis (Fig. 1). It is important to notify that the only two major proteins in the ACBP containing fractions of the last gelfiltration step are FABP and ACBP (Fig. 1). This means that previous work on the effect of FABP on acyl-CoA metabolism with FABP preparations only based on gelfiltration chromatography should be reevaluated because they are bound to contain ACBP. ACBP contaminations in FABP can also be a problem, if an ion-exchange chromatography step is used in the purification of FABP, especially if rat liver FABP is purified on a Q-sepharose HP column (unpublished results).

The molecular weights of rat and bovine liver ACBP have been determined by plasmadesorption time of flight mass spectrometry to be 9932 ± 10 and 9956 ± 10, respectively. The respective molecular weights calculated from the sequence were 9937.8 and 9955.3 [7]. These results show that the protein contains no modified amino acids apart from the N-terminal acetate group.

The sequence of bovine and rat liver ACBP (Fig. 2) was determined by Edman degradation [8, 17]. This sequence has been confirmed on bovine brain ACBP (DBI) both by Edman degradation and

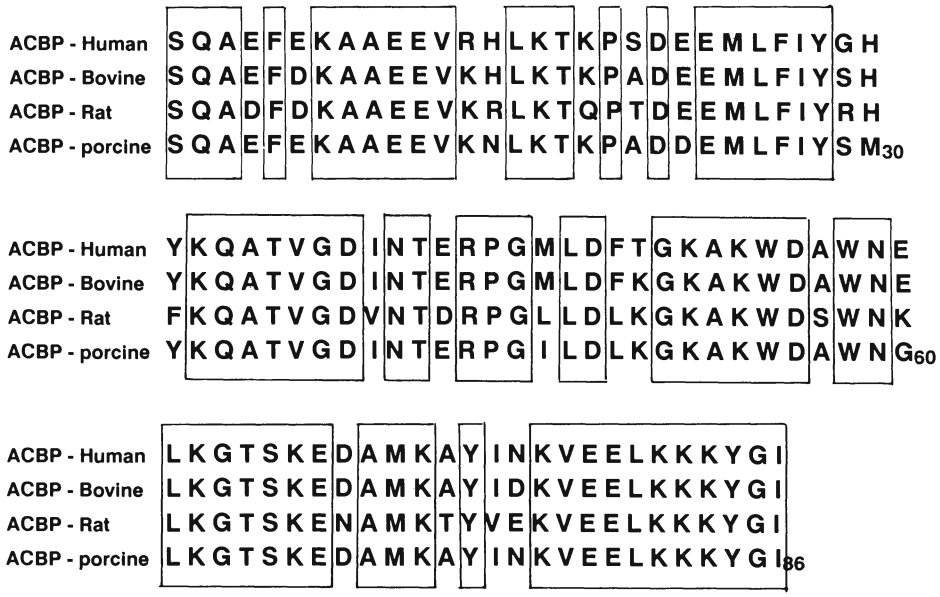

Fig. 2. Sequence comparison of ACBP (DBI) from bovine liver [17], rat liver [8], human brain [19, 21] and porcine intestine [22].

cDNA sequencing [18, 19] and on the cDNA level for rat brain ACBP (DBI) [15, 21]. Figure 2 also shows the sequence of human brain ACBP (DBI) [19, 21] and porcine intestine ACBP (DBI) [22]. It can be seen that ACBP is a highly conserved protein, 76% of the sequence being identical when ACBPs from the four species are compared. A large part of the differences represents conservative changes which will not influence structure.

Ligand binding characteristics and hydrophobic binding site

Binding specificity of bovine and rat ACBP for acyl-CoA esters of different chain length was determined by tyrosine fluorescence quenching at 332 nm [8, 12]. Acyl-CoA esters with a chain-length from C_8–C_{16} are bound with nearly equal and high affinity and the binding stoichiometry was estimated to 1 mol acyl-CoA/mol ACBP [8]. The binding stoichiometry has been confirmed using the Lipidex binding assay [23]. In the latter study the apparent Kd for acyl-CoA binding to bovine and rat ACBP was determined to 0.14 μM and 0.13 μM, respectively, for oleoyl-CoA, and 0.22 μM and 0.28 μM respectively for palmitoyl-CoA. These results indicate that ACBP has a higher binding affinity for acyl-CoA esters of unsaturated than of saturated fatty acids. ACBP binds acetyl- and butyryl-CoA only with low affinity, furthermore ACBP does not bind fatty acids [8, 12, 23]. It can therefore be concluded that both the CoA and the acyl moiety of the acyl-CoA esters are involved in binding. The binding site must therefore contain a hydrophobic pocket and a positively charged CoA binding site. Preliminary work with photoreactive acyl-CoA esters shows that amino acids 29–38 are involved in forming the hydrophobic binding pocket (unpublished results).

Cellular and tissue distribution and concentration

More than 80% of the ACBP present in liver cells was recovered in the 100.000 g cytosolic fraction indicating that ACBP is a cytosolic protein [8, 12]. The possible presence of ACBP in subcellular organels like mitochondria and nuclei awaits further immunohistochemical studies which are in progress. The highest concentrations of ACBP are found in bovine and rat liver and amount to 3.3 and

6.4 μg/mg soluble protein, respectively. Kidney from rat and cattle both contain 1.4 μg ACBP per mg soluble protein. A similar concentration was found in bovine small intestine. The concentration in other tissue was lower, but ACBP was found in all tissues tested [19]. The latter is further supported by detection of DBI mRNA and immunoreactivity in a large number of tissues including brain [13].

The relative affinity of ACBP and FABP for binding acyl-CoA esters

FABP has been suggested to play an important role in the metabolism of long-chain acyl-CoA esters (6 for review). The appearance of a new acyl-CoA-binding protein like ACBP immediately creates the question: Which protein is important protein in acyl-CoA metabolism: ACBP or FABP? Two important questions in this connection are: 1. How is the concentration of the two proteins relative to each other and relative to the acyl-CoA concentration found in the cell?, and 2. What are the relative affinities of ACBP and FABP for long-chain acyl-CoA esters?

The FABP concentration in rat liver is about 5-fold higher than the ACBP concentration [8]. Therefore, ACBP needs to exhibit a much higher affinity for acyl-CoA esters than FABP in order to play a role in acyl-CoA metabolism.

We have carried out a number of experiments to compare the affinities of bovine hepatic FABP (hFABP) and cardiac FABP (cFABP) with ACBP for binding of acyl-CoA esters. This included Lipidex binding assay, liposome binding assay, gelfiltration chromatography and competition experiments with biological membranes [23]. These experiments clearly showed that ACBP has a much higher affinity for acyl-CoA esters than does FABP.

Both types of FABP were unable to extract measurable amount of acyl-CoA esters from multilamellar liposomes whereas ACBP under identical conditions effectively extracted acyl-CoA esters from the same liposomes. The binding of acyl-CoA esters by both types of FABP in the Lipidex assay was so low that no meaningful values of Kd could be calculated, whereas ACBP effectively bound acyl-CoA esters with a Kd of 0.1–0.2 μM. When a mixture of 10 nmoles of bovine ACBP and bovine hFABP and 5 moles of palmitoyl-CoA was separated on a Sephadex G-50 column palmitoyl-CoA appeared in the ACBP peak only. When the experiment was carried out with palmitic acid instead of palmityl-CoA, fatty acid was found in the hFA BP peak only. Finally it could be shown that bovine ACBP almost completely prevented acyl-CoA-binding to heat denaturated microsomal membranes when added in equimolar amounts to the acyl-CoA esters. Bovine hFABP had only a modest effect on acyl-CoA-binding to the microsomal membranes [23]. No quantitative measure can be given to show the difference in binding affinity of the two FABPs and ACBP because the difference of affinities is too large to be measured in the binding assay used. The above mentioned experiments clearly demonstrate that ACBP has a so much higher affinity for acyl-CoA esters that these metabolites *in vivo* most likely will be bound to this protein and not to FABP.

The concentration of ACBP in rat liver is about 400 nmoles/g protein [8]. This is 2 to 4-fold the reported acyl-CoA concentration of 108–248 nmoles/g protein [24]. Therefore both the affinity of ACBP for acyl esters and the concentration found in liver qualify ACBP to be the most important binding protein in acyl-CoA metabolism in liver.

Previously published experiments on the role of FABP in acyl-CoA metabolism in the light of the existence of ACBP

Low extraction of oleoyl-CoA by rat cFABP from multilamellar liposomes has previously been reported [25]. However, these authors found that rat hFABP was able to extract acyl-CoA from multilamellar liposomes. We have been unable to detect any significant difference in the ability of bovine cFABP and hFABP to extract acyl-CoA esters from liposomes [23]. The explanation for the dis-

crepancy between our result and the previously published results [25] could either be due to bovine hFABP and rat hFABP being different or to the preparations of rat hFABP used by these authors having been contaminated with ACBP. In our experience it is difficult to separate completely the two proteins by the methods used by this group. Furthermore these authors did provide no documentation for purity of the FABP preparations used. They also compared rat cFABP and hFABP in the Lipidex assay and here again they found the same difference between hFABP and cFABP as observed in the liposome assay. A serious complication in comparing the data obtained with the Lipidex assay by these authors and the data obtained with the same assay in our experiment [23] is a significant difference in the assay conditions used. We have worked in the concentration range 0–1.0 μM acyl-CoA and a minimum Lipidex/acyl-CoA ratio of 1000 μl/nmol. The above mentioned authors used 0–20 μM acyl-CoA and a minimal Lipidex/acyl-CoA ratio of 10 μl/nmole at the highest acyl-CoA concentration. When we used a Lipidex/acyl-CoA ratio lower than 1000 μl/nmole the Lipidex could not remove all acyl-CoA from the incubation in the blank experiments without proteins added. We therefore studied high-affinity binding only, whereas Burrier *et al.* [25] studied high- and low-affinity binding, due to the presence of free acyl-CoA in the incubation after removal of Lipidex by centrifugation. The explanation of the difference between the binding ability of rat cFA BP and hFABP in experiments reported by these authors could either be that hFABP is different from cFABP with regard to low-affinity sites or that hFABP is contaminated with ACBP.

Burrier *et al.* [25] also claimed that rat hFABP contains two independent binding sites, one for fatty acid and one for acyl-CoA. However, other investigators have been unable to show the existence of two independent binding sites [26, 27]. In contrast to earlier reports [28, 29] palmitoyl-CoA was found to bind to both rat and human hFABP with lower affinity than palmitic acid [27, 30]. However, Mishkin & Turcotte [29] only used gelfiltration in the purification of FABP. Their FABP preparation therefore will have contained ACBP. From the above discussion it can be concluded that FABP has a lower affinity for acyl-CoA than does ACBP and that the high affinity of FABP found in previously published experiments is most likely due to ACBP contamination.

It has been shown that hFABP can compete with microsomes [31] and mitochondria [32] for binding of exogenously added acyl-CoA. It has also been shown that acyl-CoA synthetase is stimulated by addition of fatty acid-binding protein [25, 34, 35]. Since this enzyme is inhibited by its product the stimulation by FABP was suggested to be due to release of product inhibition [25]. It has also been shown that FABP protects acyl-CoA against hydrolysis by membrane-bound enzymes [25, 35].

In none of the above experiments documentation for the purity of the FABP preparation used has been given. In some of the experiments FABP was only partly purified by gelfiltration chromatography [34, 35]. Some authors have extended purification by negative absorption on a CM-cellulose ion-exchanger [32, 33]. The first group is bound to have ACBP in their FABP preparations and the second might have. Furthermore it needs a good electrophoretic system with high gel concentration (20%) to separate ACBP and FABP on a sodium dodecylsulphate polyacrylamide gel, and therefore apparently pure preparations on 10–15% polyacrylamide gels might have been contaminated with ACBP.

The above strongly indicates that a great number of previously published results on the effect of FABP on acyl-CoA metabolism needs reevaluation and to be repeated with FABP preparations shown to be free of ACBP.

Conclusion

ACBP binds acyl-CoA esters with much higher affinity than do both bovine cFABP and hFABP. As the concentration of ACBP in liver is 2 to 4-fold the acyl-CoA concentration it must be expected that ACBP and not FABP is the most important transport protein for acyl-CoA in liver. A lot of

previous data on the effect of FABP on acyl-CoA metabolism must be taken with caution due to possible contamination with ACBP.

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Liver fatty acid-binding protein in two cases of human lipid storage

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Key words: FABP, L-FABP, Reye's syndrome, multisystemic lipid storage, fatty liver

Summary

FABPs in the various tissues play an important role in the intracellular fatty acid transport and metabolism. Reye's syndrome (RS) and multisystemic lipid storage (MLS) are human disorders characterized by a disturbance of lipid metabolism of unknown etiology. We investigated for the first time L-FABP in these two conditions. Affinity purified antibodies against chicken L-FABP were raised in rabbits, and found to cross-react specifically with partially purified human L-FABP. L-FABP content in liver samples of two patients with RS and MLS was investigated by immuno-histochemistry, SDS-PAGE and ELISA. L-FABP immuno-histochemistry showed increased reactivity in the liver of RS patient and normal reactivity in MLS liver. L-FABP increase in RS liver was confirmed by densitometry of SDS-PAGE and ELISA method. By these two methods the increase amounted to 180% and 199% ($p < 0.02$), respectively, as compared to controls. A possible role of L-FABP in the pathogenesis of RS is discussed.

Introduction

The past decade has seen a growing interest on the family of binding proteins, of which the fatty acid binding proteins (FABPs) are important members [1]. So far the bulk of papers was concerned with the biochemical and physiological aspects of FA BPs in the various tissues and species. The important role of these proteins in lipid metabolism, their high intracellular concentration and their selective tissue distribution, suggest their possible involvement in some human pathology, in particular in those disorders in which a defective handling of intracellular neutral lipids is observed.

Reye's syndrome (RS) is a condition characterized by acute encephalopathy associated with visceral fatty degeneration; the cause is still unknown, but its occurrence has been often observed after an acute viral illness of salicylate ingestion. The syndrome, prevalent in children but observed also in adults [2], presents with vomiting, cerebral oedema (fatal in one third of cases), acute hepatocellular demage (increase in serum transaminases), increased blood ammonia, decrease of clotting factors. Short and medium-chain fatty acids and urinary C6-C12 dicarboxylic acids and acylcarnitines are also increased [3]. The most important histological alteration is a prominent liver fatty change. Both clinical, pathological and laboratory data seems to indicate a generalized mitochondrial injury, with a block of fatty acid oxidation, and peroxisomal proliferation [4].

Multisystemic lipid storage disease (MLS) (Chanarin disease) [5] is a rare syndrome characterized by congenital ichthyosis, lipid storage in liver, gastrointestinal epithelium, cultured fibroblasts, muscle and leukocytes (Jordans' anomaly); the stored material is non-membrane bounded and it is composed by neutral triglycerides. The syndrome is transmitted as a autosomal recessive trait and it is

particularly frequent in the east mediterranean area. These patients show generalized increase of intracellar triglycerides, increased oleate and palmitate uptake, normal carnitine and acyl-carnitine transferases, acid and neutral lipase, beta-oxidation enzymes, and acetyl-CoA carboxylase activity.

Both syndromes are of unknown etiology, and have in common the lipid intracellular accumulation, acute in one, chronic in the other. For both, the determination of FABP content in affected tissues is of interest, considering its central role in the intracytoplasmic handling of lipids. In this study we analysed for the first time in human pathology the L-FABP content in the liver of two patients, one with RS and the other with MLS. We found a striking increase of L-FABP content in RS, and a normal level in MLS liver.

Experimental procedures

Patients

Case I. A 16 year-old boy with a 20 days history of fatigue and malaise, developed acute vomiting and coma while sailing. He died 18 hours before landing. Acute hepatitis was excluded. The autoptic examination showed enlarged liver (2800 gr) with macrovescicolar fatty degeneration, lipid storage in muscle and other tissues, and cerebral oedema. A diagnosis of Reye's syndrome (RS) was entertained on the basis of the history and pathological changes. Specimens of frozen or paraffin-embedded autoptic liver were obtained for FABP study.

Case II. A 51 year-old women of sicilian origin, born from two first cousins, presented congenital ichthyosis, myopathy at age 41, hypoacusia, periferal neuropathy, hepatomegaly. Blood and bone marrow smear showed non membrane bounded lipid vacuoles in white cells (Jordans' anomaly). Muscle, skin, and liver biopsy confirmed the presence of multisystemic intracellular lipid storage. The lipid storage was also confirmed in the patient's cultured fibroblasts, in which a 180% increase of oleate and palmitate uptake was observed. Carnitine, carnitine-palmitoyl transferase, beta oxidation enzymes, and neutral lipase were all within normal ranges.

Specimens of liver biopsy were snap-frozen and paraffin-embedded for FABP analysis.

Samples from 3 patients not presenting liver disfunction obtained during laparotomy, and samples of 3 autoptic livers with no pathological changes were used as controls.

FABP antibodies

Purified chicken L-FABP and partially purified human L-FABP were kindly provided by Dr Spadon [6]. Antibodies were raised in rabbits and affinity chromatography purified. Cross reactivity with human L-FABP was verified on partially purified human L-FABP by immuno-blot (Fig. 1).

Histology and immuno-histochemistry

Frozen sections and paraffin sections of patients and control tissues were stained with H&E, Oil-red O (ORO) (specific for neutral lipids). Sections were incubated with FABP at 1:25 dilution and the specific reactivity was revealed by a secondary donkey anti rabbit biotinylated antibody and by fluoresceinated streptavidin (Amersham, England).

SDS-PAGE and immunoblot

Frozen tissues were homogenated in 1:20 K-Phosphate 10 mM, NaCl 154 mM pH 7.4, centrifuged 10 min at 600 g, and the surnatant was spun 90 min at 100,000 g. The cytosolic fraction was aspirated avoiding contamination by floating fat, and stored at $-80°$ C. All steps were carried out at $+4°$ C. Protein content was evaluated by Lowry's method, and SDS-PAGE was performed essentally according to Laemmly with 16–20% gradient gels. Proteins were stained with Coomassie brilliant blue or blotted on nitrocellulose paper (BA-85, Schleicher & Scull, Dassel, FRG) and revealed by anti L-FABP antiserum diluted 1:200 and alkaline phosphatase conjugated antirabbit IgG (Sigma Chemical Co. St. Louis, MO).

Densitometry

Gels stained with Coomassie brilliant blue were scanned at 595 nm in EDL densitometry (Helena

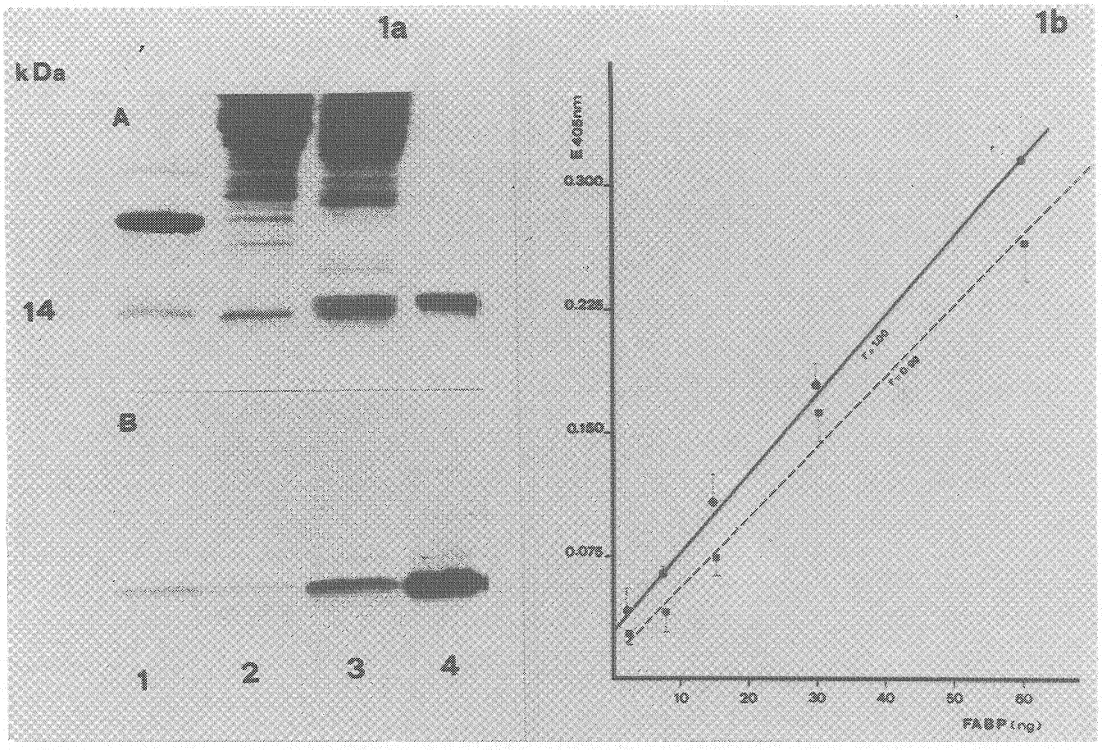

Fig. 1a. Specificity of anti L-FABP antibodies: Coomassie brilliant blue (A) and immunoblot with anti-chicken L-FABP antibodies (B) of 15% SDS-PAGE. 1) Human partially purified L-FABP (6 μg); 2) Human liver cytosol (22 μg); 3) Chicken liver cytosol (18 μg); 4) Purified chicken L-FABP (8 μg). *Fig. 1b.* Calibration of ELISA with purified chicken L-FABP (circles), and partially purified human L-FABP (squares).

Lab. Beaumont, TX). The results were expressed as percent of total stained proteins.

ELISA

A standard curve was obtained with 0–60 μg partially purified human or chicken L-FABP (Fig. 1) absorbed on polystyrene wells (Maxisorb V96 plates, Nunc, Roskilde, Denmark). The amount of immunoreactive protein in patients and controls was evaluated by regression analysis of the O.D. at 405 nm in a Titertek Multiskan MCC (Flow laboratories, McLean, VA).

Results

H&E and ORO stains confirmed in both patients the liver fatty changes which were more prominent in the RS case (Fig. 2). Immuno-histochemistry showed good cytoplasmic reactivity in all specimens, but the intensity of the signal was much higher in the case of RS compared to both control and MLS case (Fig. 2). No difference was seen between MLS and control.

SDS-PAGE showed a clear band of about 14 kDa Mr in all samples, corresponding to the purified chicken L-FABP, which was more intense in the liver of RS (Fig. 3). The 14 kDa band represented 7.02% of cytosolic proteins in bioptic control liver, and 6.90% in MLS liver. In autoptic controls the band was the 11.01%, and in RS liver it was the 19.8%.

The increase of L-FABP in RS autoptic liver was confirmed by ELISA: L-FABP in RS patient liver was 3.71 ± 0.47 μgr/mg of cytosolic protein while in controls it was 1.86 ± 0.59 μgr/mg of cytosolic

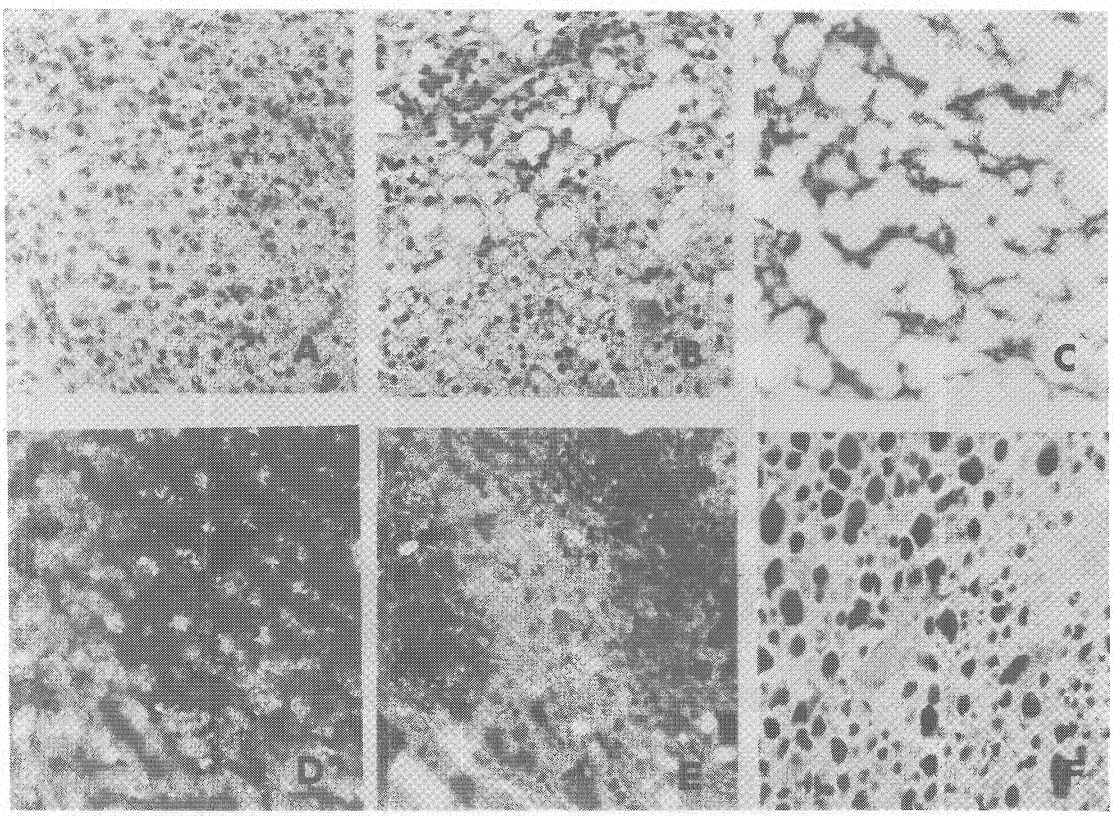

Fig. 2. Liver frozen sections: A-B-C (H&E × 200) show evident vacuolization in hepatocyotes of Multisystemic lipid storage (B), and Reye's syndrome (C) versus control (A); D-E-F immuno-histochemistry with anti L-FABP antiserum × 200: immunoreactivity is much stronger in Reye's syndrome (F) vs control (D) and Multisystemic lipid storage (E).

protein ($p < 0.02$). In face of this L-FABP increase, patient liver showed lower protein content per mg of wet tissue (mg-ww) compared to controls. L-FABP content expressed as μg/mg-ww was the same in patient and controls (124.66 vs 124.77 μg/mg-ww).

Discussion

In the present study we investigated L-FABP content in the liver of two patients with lipid storage of unknown etiology. The pathologic picture (liver steatosis), and the biochemical data (impaired fatty acid utilization in RS, and a disorder of intracellular lipid homeostasis in MLS) were suggestive of a possible involvement of FABP in the pathogenesis of these two conditions. The most striking result is the marked increase in reactivity to anti-FABP antibodies observed in the liver of RS patient by immuno-histochemistry. The presence of a strong 14 kDa band in SDS-PAGE of the RS liver cytosol, and his quantitation by ELISA, confirmed a consistent increase of L-FABP in this pathology (180% and 199% increase vs controls). The use of anti-chicken L-FABP for ELISA might decrease the sensitivity of the test. Studies with anti-human L-FABP antibodies (kindly provided by Prof. J.H. Veerkamp) are now in progress to increase the sensitivity of this evaluation.

The protein content per mg-ww was much lower in the RS patient sample (50% of normal), and therefore this raises the question of the specificity of L-FABP increase. The total liver weight was increased in our patient due to the high lipid content, and this fact alone can explain the lower pro-

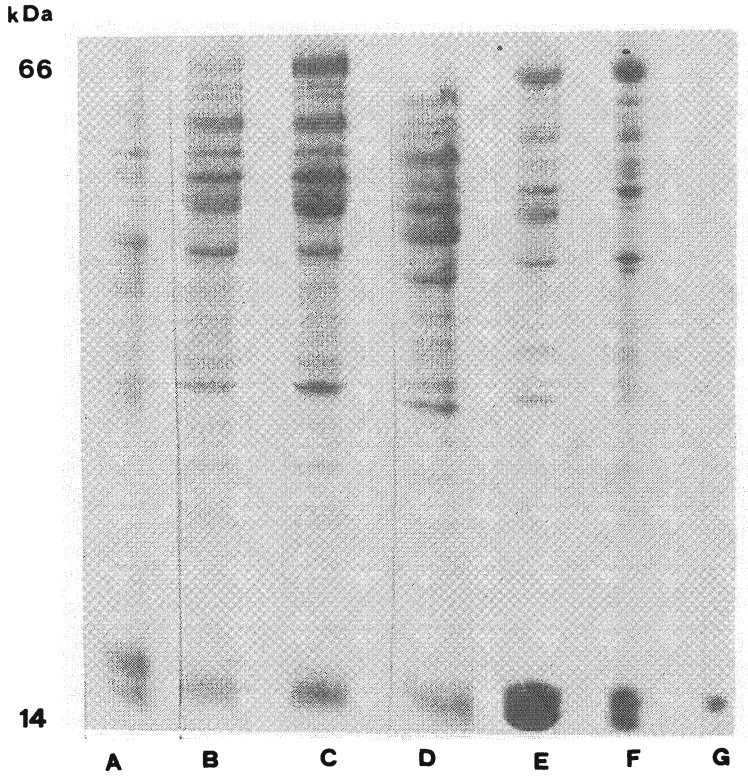

Fig. 3. 16–20% SDS-PAGE of A) chicken liver cytosol (40 μg); B-C) human control liver cytosol (40–50 μg); D) MLS liver cytosol (50 μg), E-F) RS liver cytosol (40 – 20 μg); G) Chicken purified L-FABP (3 μg). Coomassie brilliant blue.

tein content per gram of wet tissue. Further studies on more RS patients and on patients with liver fatty change from other causes (toxic, infectious, metabolic) are now needed to ascertain the specificity of the FABP change observed in our case.

We advance the following hypothesis on the role of L-FABP increase in the etiopathology of RS. A gradual increase in L-FABP has been reported during postnatal development in the rat [7], higher L-FABP is found in rat female as compared to male [8], increase in L-FABP can be induced in rat by ethanol [9], tiadenol and clofibrate [10]. While the first two cases reflect physiological changes and hormonal status, in the two latter models there is a perturbation of fatty acids metabolism with proliferation or activation of liver peroxisomes. Liver peroxisomes proliferate in RS, probably in response to the block of mitochondrial beta-oxidation and to the activation of omega-oxidation [11]. L-FABP could be needed in this contest for the maintenance of fatty acids homeostasis. It has been shown [12] that the increase of FABP concentration above 3 g/l in rat heart mitochondria suspension has a deleterious effect on beta-oxidation. Even though it is not certain that L-FABP shares with the heart FABP the property of a beta-oxidation regulator, the following pathogenetic hypothesis could be suggested for RS: a still unknown agent (and possibly not a single one) causes a liver mitochondrial injury with partial block of beta-oxidation, there is an activation of the alternative pathways of peroxisomal beta-oxidation (with production of toxic medium chain-acylCoA's, partially buffered by carnitine) and omega-oxidation (with production of dicarboxylic acids), with a concomitant increase in L-FABP in order to handle the surplus of fatty acids; the increase in L-FABP and dicarboxylic acids eventually causes a further mitochondrial beta-oxidation block, and the accumulation of medium-chain acylCoA further damages

cell membranes: a vicious circle is now operating, leading to progressive cellular damage. Therefore in RS L-FABP could first operate as part of a compensatory mechanism, and then participate to the mitochondrial oxidative block.

MLS showed normal L-FABP content. It is not yet clear whether this disorder arises from increased cellular uptake, increased synthesis, altered compartimentation, or defective lipolysis [5]. The first three hypothesis are attractive, and should be further investigated by evaluating in this and other patient liver or fibroblasts the plasma membrane FABP [13] or the acyl-CoA binding protein [14].

In conclusion, our studies demonstrate a significant increase of L-FABP in RS autoptic liver, and a normal content in MLS bioptic liver. L-FABP appears to have a possible role in the pathogenesis of RS.

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Nomenclature of fatty acid-binding proteins

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Key words: nomenclature of proteins, fatty acid-binding protein, lipid binding protein

Summary

A variety of designations is currently being used to refer to cellular fatty acid-binding proteins (FABPs). Besides from the use of other general names (e.g. Z protein), confusion mostly arises from the application of various abbreviations and symbols to denote the tissue(s) of origin and cellular localization (cytoplasm, plasma membrane) of a specific FABP. In order to minimize confusion a more unified and rational nomenclature is proposed, which is based on application of the formula X-FABP$_Y$. The prefix X is a capital letter indicating the tissue of greatest abundance, the suffix Y similarly denotes the (sub)cellular localization of the protein. The general and functional name 'fatty acid-binding protein' (FABP) is preferred for the cellular proteins with the property to bind fatty acids, unless future research reveals that the binding of fatty acids is not the primary biological property or physiological role of (some of) these proteins

Introduction

In 1972 Ockner and coworkers [1] discovered a class of mammalian cytoplasmic proteins by their ability to bind *in vitro* long-chain fatty acids, and designated these accordingly as fatty acid-binding proteins (FABP). Subsequent research in several laboratories revealed FABP to be identical to a cytoplasmic anion-binding protein previously identified in liver and called protein A by Ketterer *et al.* [2] and Z protein by Levi *et al.* [3]. In addition, more recently it was established that FABP from liver cytoplasm is identical to heme binding protein [4] and to the mitosis-associated p14 protein [5]. (The identity with sterol and squalene carrier protein (SCP) isolated by Dempsey and coworkers has been discussed elsewhere; see ref. 6.) Conversely, an ileal cytoplasmic protein initially called gastrotropin now appears a distinct fatty acid-binding protein [7]. Although these proteins are currently referred to mostly as FABP, the other above-mentioned names are still also widely used. (The term Z-protein is especially confusing as it also refers to a tetrameric (55 kDa subunits) Z-line component found in chicken striated muscle [8].)

The number of designations used to describe this protein did further increase when the existence of tissue-specific types of FABP and of isoforms of one type became apparent [9]. Moreover, another fatty acid-binding protein, distinct from cytoplasmic FABP, was recently found to be associated with the plasma membrane [10]. The FABPs are generally named after their tissues of greatest abundance, a custom which in itself has hardly caused confusion. However, since the tissues are referred to in different manners, a host of abbreviations and symbols has appeared. For example, the designation H-FABP (or h-FABP) has been used to denote the FABPs both from heart cytoplasm and from liver (hepatic) cytoplasm, as well as from liver plasma membrane. Another common abbreviation for plasma membrane FABP is M-FABP which, however, may also stand for (heart) muscle FABP from cytoplasmic origin.

The existence of this multitude of designations clearly calls for the need of a uniform and prefer-

ably rational nomenclature, especially with respect to abbreviations and symbols used. On the occasion of the First International Workshop on Fatty Acid-Binding Proteins, held in Maastricht, the Netherlands, in September 1989, the problems outlined above were discussed and provisonal agreement was reached on a recommended nomenclature for FABPs.

Subclasses of fatty acid-binding proteins

Each protein that exhibits affinity for the non-covalent binding of long-chain fatty acids can be designated as a fatty acid-binding protein. The best known representative of this class of proteins is serum albumin (68 kDa), which derives its trivial name from a physical appearance similar to that of the earlier known water-soluble proteins found in egg-white and milk (now called ovalbumin and lactalbumin, respectively) (cf. albus, white; albumen, white of egg). The other presently known examples, being those proteins isolated from plasma membranes (40 kDa) and those from cytoplasm (13–15 kDa), have not (yet) been given a trivial name and hence are referred to each as fatty acid-binding protein, thus disclosing their putative main physiological function. For the cytoplasmic FABP other trivial names have been suggested, e.g. lipmodulin [11], but general preference is given to an abbreviated form of a name that expresses the main biological property of the protein. Since the latter is not yet known in much detail [9], the term 'fatty acid-binding protein' is recommended for continued use. However, as soon as more knowledge is obtained on its biological role(s), a revision of the names should be considered. To this end several proposals have already be made, e.g. fatty acyl-L-carnitine binding protein (also abbreviated as FABP) [12], fatty acid-transfer protein (FATP), fatty acid translocase (FAT), and the more general term (cellular) lipid binding protein (LBP) [13]. Similarly, the plasmalemmal FABP is recommended to be renamed (into e.g. fatty acid receptor or fatty acid translocator) only when given rise to by new insights into its nature and physiological significance.

Types and isoforms of cytoplasmic fatty acid-binding proteins

Several cytoplasmic FABP types have now been identified [9, 14] and each is named after the tissue of greatest abundance. Being the product of a separate gene, each FABP type has a distinct amino acid sequence and length (within the range of 120 to 134 residues). FABPs found in the same tissue and differing in only a few amino acid residues are referred to as isoforms within a type [14]. Note that FABPs with identical polypeptide chains but differing in amount and/or type of bound ligands are designated as apo- and holoforms (not isoform) of the protein (with the apo-form being the ligand-free protein).

Suggested nomenclature

To unambiguously indicate the subclass and type of a particular FABP or to give its full biological origin in case of a FABP not earlier described, it is recommended to apply the following formula:

$$X\text{-}FABP_Y$$

in which the prefix X denotes the tissue of origin or of greatest abundance, to be indicated by the first (capital) letter of the regular English name of that tissue, and in which the suffix Y denotes the (sub) cellular localization of the protein (C for cytoplasmic, PM for plasma membrane, MI for mitochondrial, N for nuclear etc.). In practice, a consequent use of these suffixes will be necessary only in texts dealing with more than one subcellular FABP. Capital letters are preferred (i) for analogy, e.g. with the tissue related nomination of creatine kinase isozymes, and (ii) since lower-case letters already have several other meanings for example in cDNA, mRNA, rRNA, cAMP.

In Table 1 the recommended abbreviations are given for the distinct proteins now identified, together with some of the most common other designations used in literature. It should be noted that for the time being heart muscle $FABP_C$ should be abbreviated as $H\text{-}FABP_C$, so that the term M-

$FABP_C$ is reserved for the protein from skeletal muscle. In case the two FABPs appear to be identical the term M(uscle)-$FABP_C$ may be adopted. Gastrotropin could be included in this system, by virtue of its presence in the ileum, as I(leal)-FA BP_C, but this would require the well-characterized I(ntestinal)-$FABP_C$ to be renamed, e.g. into J(ejunal)-$FABP_C$.

It has also been suggested to include in the above formula a one-letter symbol to indicate the species origin of the protein. However, bearing in mind that clarity and unambiguity are more important than brevity [15], the addition of more symbols should be avoided. Thus, it is recommended to give the full species name preceding the formula.

Since the existence of specific isoforms has not (yet) been found to be a common feature of FABP types, for these no recommendations for terminology are given (cf. ref 14).

Related terminology

The 13–15 kDa FABPs are currently called either cytosolic (or (cyto)soluble) proteins, or cytoplasmic proteins. However, the non-identity of these terms should be kept in mind [16]. The biochemical term 'cytosol' refers to the soluble phase (105,000 × g supernatant) of cell extracts, which contains essentially components of the aqueous cytoplasm (i.e. the interorganelle cytoplasm) of intact cells, but may also contain compounds easily released from other subcellular compartments as well as interstitial components. Above all, 'cytosol' cannot be taken as a subcellular compartment. Therefore, the biological term '(aqueous) cytoplasm' may be recommended for general use, as it can well be applied in most cases, whereas the term 'cytosol' can be applied in a strictly biochemical context only.

Since many of the papers on FABPs deal with studies on their binding properties for fatty acids and derivatives, the reader's attention is also drawn to a proper use of fatty acid nomenclature [17, 18].

Concluding remarks

The existence of at least six (but probably more) distinct types of cytoplasmic FABP (Table 1) makes this subclass of proteins rather unique, but at the same time presents us with the problem of plainly nominating each of them. Analogous issues from related areas of research are scarce. Perhaps the best example is the family of phospholipid transfer proteins, in which individual members ex-

Table 1. Nomenclature of 'fatty acid-binding proteins'[1]

FABP subclass and type[2]	Recommended abbreviation	Other common designations
Cytoplasmic FABP	$FABP_C$	
Liver	L-$FABP_C$	Z, h-FABP
Intestinal mucosa[3]	I-$FABP_C$	g-FABP, i-FABP
Heart muscle	H-$FABP_C$	c-FABP, h-FABP
Adipose tissue	A-$FABP_C$	p422, aP2, a-FABP, ALBP[4]
Brain	B-$FABP_C$	b-FABP
Kidney	K-$FABP_C$	r-FABP
Skeletal muscle[5]	M-$FABP_C$	
Plasma membrane FABP	$FABP_{PM}$	M-FABP, PM-FABP
Liver[5]	L-$FABP_{PM}$	L-FABP, LPM-FABP
Heart[5]	H-$FABP_{PM}$	h-$FABP_{PM}$

[1] Note the hyphen, recommended to facilitate literature searches.
[2] Type denotes the tissue of greatest abundance.
[3] Excluding gastrotropin (see text).
[4] Adipose lipid binding protein.
[5] Identification as a distinct type neither established nor excluded.

hibit a unique preference for specific phospholipids so that the acquired nomenclature is based on this property, e.g. PC-TP stands for phosphatidylcholine-specific transfer protein [19].

The presence of one type of cytoplasmic FABP in more than one tissue, as was found for L-$FABP_C$ and H-$FABP_C$, might make the use of a prefix denoting to a tissue rather irrational. However, since in these two cases the prefix still indicates the tissue of greatest abundance, this custom has met general approval. Hence, for lack of other useful criteria, the distinction on the basis of tissue occurrence was hold on in the formulation of the presently recommended nomenclature. For the same reason, the FABPs associated with mitochondria and nuclei should, for clarity, be designated as $FABP_{MI}$ and $FABP_N$, respectively, unless their presumed identity with $FABP_C$ [14] is confirmed, so that the latter designation may replace these terms.

Recently it was established that L-$FABP_C$ is identical to heme binding protein (HBP) [4] and to the mitosis-associated p14 protein [5]. In addition, functioning of L-$FABP_{PM}$ as a glutamic oxaloacetic transaminase has been suggested [20]. These new findings illustrate that it is not excluded that the principal function of a certain FABP may ultimately appear to be different, whereby its fatty acid-binding property should be regarded as a secondary phenomenon. Obviously, such development would then implicate a change of nomenclature for that particular FABP.

In summary, in this report a recommended nomenclature for fatty acid-binding proteins is presented, which agrees to a large extent with the currently applied terminology (cf. notes on nomenclature in refs. 6, 21 and 22), but in which confusing issues, especially with regard to the use of prefixes and suffixes to denote the origin of the protein, are eliminated. Because of our as yet limited knowledge on the physiological significance of FABPs options for other recommendations are kept open. It is hoped that on an interim basis this more uniform nomenclature may help to minimize confusion and thus to facilitate the exchange of knowledge on this intriguing family of proteins.

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Cellular fatty acid-binding proteins: current concepts and future directions

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Key words: lipid transport, fatty acid-binding protein, acyl-CoA binding protein

Summary

At least three different proteins are implicated in the cellular transport of fatty acid moieties: a plasmalemmal membrane and a cytoplasmic fatty acid-binding protein ($FABP_{PM}$ and $FABP_C$, respectively) and cytoplasmic acyl-CoA binding protein (ACBP). Their putative main physiological significance is the assurance that long-chain fatty acids and derivatives, either in transit through membranes or present in intracellular compartments, are largely complexed to proteins. $FABP_C$ distinguishes from the other proteins in that distinct types of $FABP_C$ are found in remarkable abundance in the cytoplasmic compartment of a variety of tissues. Although their mechanism of action is not yet fully elucidated, current knowledge suggests that the function of this set of proteins reaches beyond simply aiding cytoplasmic solubilization of hydrophobic ligands, but that they can be assigned several regulatory roles in cellular lipid homeostasis.

Introduction

Translocation of lipophilic compounds through aqueous compartments is generally considered to be facilitated by specific proteins. In plasma and interstitium albumin is the main transport vehicle for many of these substances, but several other, more specific extracellular lipid binding proteins are also known, e.g. for the transfer of vitamins A and D, cholesterol, cholesteryl esters, steroid hormones etc. Proteins possibly involved in lipid transport inside mammalian cells have been studied only during the last 20 years, initiated by the pioneering work of Levi and colleagues [1] on the role of cytoplasmic proteins in the hepatic uptake of bilirubin and other organic anions. Simultaneous reports on the requirement of cytosolic factors for the microsomal synthesis of cholesterol [2], and for the transfer of phospholipids between membranes [3] also gave strong impulses for intensive investigations on so far unknown cytoplasmic lipid transfer and carrier proteins.

At present several families of cellular proteins involved in the metabolism of lipophilic compounds have been identified (for an excellent review see ref. 4; and see ref. 5). These are (i) the group of so-called Y proteins of about 30–50 kDa, to which belong the glutathion-S-transferase (ligandin) family, a number of specific bile acid-binding proteins and the plasmalemmal fatty acid-binding protein; (ii) the family of phospholipid binding proteins (18–26 kDa); (iii) the family of glycolipid binding and transfer proteins (20–27 kDa), and (iv) a group of proteins of about 9–17 kDa (the so-called cytosolic Z fraction) including, among others, cytoplasmic fatty acid-binding proteins, sterol carrier proteins, retinoid binding proteins and acyl-CoA binding protein. With the exception of the glutathion-S-transferases, enzymic activity of these proteins has not (yet) been observed. Structural and functional relationships among them have yet only partly be defined. Therefore, the (non-)identity of described proteins can not be fully established yet, and, in addition, the formulation of a rationale nomenclature still offers a problem.

Proteins exhibiting a high affinity for the non-covalent binding of long-chain fatty acids, accordingly referred to as fatty acid-binding proteins (FABPs), were first detected in cytosol by Ockner and co-workers in 1972 [6, 7] in studies on the intestinal absorption of long-chain fatty acids. FABPs have now become the subject of investigation in a steadily growing number of laboratories. In addition, the plasmalemmal membrane was recently found to also contain a protein capable of reversible association with fatty acids [8, 9]. The broad interests in these cellular proteins has prompted the organization of the First International Workshop on Fatty Acid-Binding Proteins, held in Maastricht, the Netherlands, on September 4 and 5, 1989. During this meeting three main aspects of cellular lipid binding proteins have extensively been discussed:

a. occurrence and tissue distribution of the cellular FABPs and of acyl-CoA binding protein.
b. physico-chemical aspects of cytoplasmic FA BPs.
c. physiological significance of cytoplasmic and plasmalemmal FABPs and the relation with acyl-CoA binding protein.

In this overview, we report the current concepts and controversial issues on cellular FABPs as presented at the 1st FABP Workshop, together with perspectives for future research.

Fatty acid-binding proteins: occurrence and tissue distribution

Proteins capable of binding long-chain fatty acids are found both in cytoplasm and also in plasmalemma of mammalian cells of virtually all tissues examined [10–15]. Since both are referred to as fatty acid-binding protein (FABP), the recent discovery of the plasmalemmal FABP, together with the identification of various types and isoforms of the cytoplasmic FABP, have caused much confusion in terminology. This aspect is more profoundly discussed in an accompanying paper which also gives recommendations for a more unified and unambiguous nomenclature [16].

Fatty acid-binding proteins are also found in plants [17, 18] but these will not be discussed here.

Cytoplasmic FABPs

The three main types of cytoplasmic FABP (FA BP_C) identified at present are liver (L-), intestinal mucosa (I-) and heart (H-)$FABP_C$, each named after the source of their first isolation. More recently, additional distinct $FABP_C$ types have been identified in adipose tissue [19–21], kidney [22, 23], placenta [24], myelin [25] and brain [26]. All FA BPs belong to an ancient gene family comprised of 10 or more structurally related cytoplasmic proteins containing 127–136 amino acid residues and binding hydrophobic ligands [7]. The family also includes the cellular retinol- and retinoic acid-binding proteins, gastrotropin and a mammary-derived growth inhibitor (MDGI). Gastrotropin appears to be a further type of FABP, which is expressed in mucosal cells of the ileum [27, 28]. Interestingly, both heart and brain FABP show a larger structural homology with MDGI [29] than with the other FABP types (cf. ref. 30).

In addition to a division into tissue-related types of $FABP_C$, isoforms of L-FABP and of H-FABP have been found in several mammalian species. The two isoforms of bovine L-FABP as well as those of bovine H-FABP differ in a single mutation only, in that in both cases Asn in one isoform is replaced by Asp in the other. This deamination results in differences in isoelectric point and, with the liver isoforms, also in fatty acid-binding stoichiometry [30, 31]. Furthermore, when cloned cDNA of bovine H-FABP was expressed in *Escherichia coli,* up to four isoforms were produced [32]. The existence of isoforms of FABP types has also been reported for human H-FABP [33], human L-FABP [34] and the FABPs from adipose tissue [5] and human placenta [35]. FABPs obtained from the same tissue and differing in the amount and type of endogenously bound fatty acids but not in amino acid composition, have improperly also been designated as isoforms [24, 36–38]. When fully identical polypeptide chains are

involved, the terms apo-FABP and holo-FABP would better apply for distinguishing the native protein and the FABP-fatty acid complex, respectively [16].

The $FABP_C$ types each show a distinctive pattern of tissue expression with some tissues, such as intestinal epithelium and kidney, containing more than one type. The most abundant quantities of $FABP_C$ are found in liver, intestine, heart and adipose tissue (40–55 μg/mg cytosolic protein for the adult rat tissues) [39–43]. Since the average molecular weight of cytosolic proteins (based on gel filtration) is about 80–100 kDa, for these tissues FABP will present a remarkable 25–35% of the number of soluble proteins. Unlike the other types, H-FABP is considered, based on quantitative immunoassays, to be expressed in a broad range of tissues, including slow-twitch skeletal muscle, mammary gland, gastric mucosa [44], brain, testis, ovary, kidney and aortic wall [35, 40, 42]. However, although immunochemical cross-reactivity of type-specific (polyclonal) antibodies with other FABP types is negligible among L-FABP, I-FABP and H-FABP [42, 45, 46], this may not be true among other, not yet identified types of FABP and the latter ones. Thus, the suggested presence of $H\text{-}FABP_C$ in brain will presumably relate to cross-reactivity of anti-$H\text{-}FABP_C$ antibodies with the brain-type $FABP_C$, which shows 95% amino acid sequence homology with $H\text{-}FABP_C$ [26]. Similarly, the FABP found in skeletal muscle may also be closely related but distinct from H-FABP [47]. Since the fatty acid-binding capacity of cytosolic proteins from adrenals can not be accounted for by immunoreactivity with antibodies against known FABP types, the adrenal gland is thought to also contain a distinct FABP type [35].

The presence of FABP in endothelial cells is not yet clear. Using antibodies raised against H-FABP and immuno-electron microscopy Fournier and Rahim [48] detected appreciable amounts of FABP in cardiac endothelial cells. However, by quantitative immunoassay, Linssen *et al.* [49] could detect only minute amounts of H-FABP in cultured rat cardiac endothelial cells. On the other hand, with a comparable technique, Paulussen and co-workers [50] found cultured human umbilical cord artery endothelial cells to contain about one third of the concentration in human heart.

In several tissues the regional organ distribution of the FABPs has been studied either with immunohistochemical methods using type-specific (immunofluorescent or gold-labeled) antibodies [30, 35, 48, 51–56], or with *in situ* hybridization using a synthetic oligonucleotide complementary to FABP mRNA as probe [57]. Marked gradients in the expression of FABP have been found in liver and intestine, in relation to differences in fatty acid metabolism. In the liver acinus of the male rat, L-FABP is expressed in a declining gradient from the periportal to the pericentral hepatocytes. Female rats, however, do not show such an acinar gradient. In intestine, both L-FABP and I-FABP decline in abundance from jejunum to ileum and from the villus tips to the crypt cells of the intestinal villi. In kidney, H-FABP appears to be present predominantly in the distal tubules of the outer medulla, while the kidney-specific (K-)FABP was found almost exclusively in the proximal convoluted tubules [35].

Although largely confined to the soluble cytoplasm, FABP has also been immunocytochemically localized in and isolated from subcellular organelles, such as mitochondria and nuclei, of rat and bovine heart and liver [48, 52, 53], with the exception of bovine liver mitochondria [30, 52]. However, with immunochemical assays on subcellular fractions, Crisman *et al.* [40] could not confirm the intramitochondrial presence of H-FABP in rat heart. Evidence for the binding of FABP to outer-mitochondrial and microsomal membranes has been reported for bovine liver [52], but could not be established for bovine heart [30]. In hepatocytes from fed rats, FABP was found most densely accumulated around glycogen stores, whereas in fasted rats a more diffuse localization was noted [58].

$FABP_C$ is normally not found in plasma or interstitial fluid, but may be released into the blood compartment upon cellular injury e.g. of myocardial tissue during ischemia and reperfusion [59–61]. Because of its small size, cytoplasmic abundance and high degree of tissue specificity the plasma

level of $FABP_C$ types might be particularly suitable for quantitation of tissue damage in patients.

Acyl-CoA binding protein

In 1987, Mogensen *et al.* [62] first described in bovine liver a cytosolic protein with a specific binding affinity for medium- and long-chain acyl-CoA esters (not fatty acids). The acyl-CoA binding protein (ACBP) has a molecular mass of 9.9 kDa. Its amino acid sequence shows no homology with FABP or other lipid binding proteins, but appears identical with diazepam-binding inhibitor (DBI) protein isolated previously from mammalian brain [63, 64]. The physiological consequence of the identity of ACBP with DBI is further discussed elsewhere [64]. ACBP heterogeneity was not (yet) observed; the same protein is found in all tissues investigated, mainly (over 80%) in the soluble protein fraction, with highest concentrations found in liver and a 4-fold (cow) or 14-fold (rat) lower value in heart (Table 1).

Plasmalemmal FABPs

First identified in 1985 in rat liver [8, 9], a (probably identical) plasmalemmal membrane-associated FABP ($FABP_{PM}$) has now also been described in other cell types with high transmembrane fluxes of fatty acids, namely jejunal mucosal cells, adipocytes, cardiac myocytes and kidney basolateral membranes [65]. The basic (pI 9.0) membrane protein has a molecular weight of 40 kDa, lacks carbohydrate or lipid components, appears unrelated, both structurally and immunochemically, to FA BP_C, and is believed to span the entire phospholipid bilayer as integral membrane protein. Recent studies by Berk and co-workers [66] have shown that rat liver $FABP_{PM}$ is structurally closely related to the mitochondrial isoform of glutamic-oxaloacetic transaminase (mGOT). L-$FABP_{PM}$ was found to also exhibit mGOT enzyme activity. However, the two proteins are not identical, as Stremmel *et al.* [67] were able to raise monospecific antibodies directed against rat L-$FABP_{PM}$, which showed immunoreactivity with $FABP_{PM}$ in other tissues, but not with rat mGOT. Rat L-$FABP_{PM}$ is an abundant component of the plasma membrane as it is estimated to constitute 2% of plasmalemmal protein, which equals $1–3 \times 10^7$ sites per hepatocyte [66].

Physico-chemical aspects of cytoplasmic FABPs

The controlled expression of $FABP_C$ in *Escherichia coli,* as described for rat L-FABP [68] and I-FABP [69] and recently also for murine A-FABP [21], has permitted the generation of gram-quantities of pure protein so as to allow X-ray diffraction as well as high-frequency NMR spectroscopy studies. These latter techniques are powerful tools to unravel the molecular structure and ligand binding parameters, respectively, of FABP.

Structure of $FABP_C$

Structural analysis at 2.0 Å resolution of crystalline *E. Coli*-derived rat I-$FABP_C$ either without or with bound palmitate were carried out by Sacchettini and co-workers [70–73]. Both apo- and holo-I-

Table 1. Expression of $FABP_c$ and ACBP in liver and heart of adult cow and rat

Species	Tissue	$FABP_c$	ACBP
		(nmol/g ww)	
Cow	Liver	230[a]	15
	Heart	7[b]	4
Rat	Liver	160	28
	Heart	102	2

Tissue contents of the proteins (in nmol/g wet weight of tissue) were calculated from their cytosolic contents as assayed immunochemically using monospecific antibodies, and reported in μg/mg cytosolic protein in refs. 40, 52, 53, 63 and 124. For both species the overall cytosolic protein contents of liver and heart were taken as 45 and 30 mg protein/g ww, respectively [40, 63, 124]. No correction was made for the small portion (less than 15%) of total tissue $FABP_C$ or ACBP which is not recovered in the cytosol.

[a] Sum of pI-6.0 and pI-7.0 isoforms.

[b] Sum of pI-4.9 and pI-5.1 isoforms.

FABP are composed of 10 anti-parallel β-strands organized into two nearly orthogonally situated β-sheets, thereby creating a clam-like structure. The bound molecule of palmitate was localized between the β-sheets (the so-called β-barrel) in a bent conformation, completely encased by protein, similar to the β-barrel topology of P2 myelin protein [74] and of chicken liver (basic) FABP [75] (and several other binding proteins for hydrophobic ligands). Seven ordered solvent molecules were identified within the core of the protein in close proximity to the fatty acid. In the apo-protein the fatty acid-binding site contains six additional ordered solvent molecules so that there are no major conformational differences between the apo- and holo-proteins. These data suggest that fatty acid binding by FABP is accompanied by a concomitant release of solvent molecules. The carboxylate group of the fatty acid is located away from the protein/solvent interface, as it interacts in the core of the FABP molecule with residues Arg^{106} and Gln^{115}, by forming part of a unique five member hydrogen bonding network, further consisting of two ordered solvent molecules and the two amino acid side chains [73]. The terminal methyl group of the hydrocarbon chain is located more closely to the surface of the protein.

The three-dimensional structure of rat L-$FABP_C$ has not yet been determined, but evidence is available that L-FABP shows a different structural interaction with its ligands than does I-FABP. Using a high-resolution solution state NMR method, Cistola *et al.* [76–78] studied the interactions between carboxyl-^{13}C-enriched fatty acids and either *E. Coli*-derived I- or L-FABP. With this powerful non-invasive method it was found that for L-FABP, when compared to I-FABP, the fatty acid-binding environment is more solvent accessible and the carboxylate groups of bound fatty acids are located at or near the aqueous surface of the protein and involved in absent or weak electrostatic interactions [77].

Ligand binding properties

The ligand binding specificity of L-FABP is much less than that of the other FABP types, since the protein exhibits affinity for the non-covalent binding not only of long-chain fatty acids, but also of heme, lysophosphatidylcholine, bilirubin, carcinogens, prostaglandin E_1, retinoids, retinyl palmitate, fluorescent fatty acid analogues, and even gold (aurothiomalate) and selenium [10, 12, 79, 80]. This finding appears in accordance with the liver being the organ active in metabolizing diverse lipophilic compounds (although the preferential binding of heme [81, 82] could indicate that L-FABP primarily functions in intracellular heme metabolism). This binding diversity and the finding of a binding stoichiometry of 2–3 mol fatty acids/mol L-FABP (see below) had previously been interpreted to indicate that L-FABP has a single ligand binding site, larger than in other FABP types and capable of accomodating 2–3 fatty acids or various bulky hydrophobic compounds [83]. This concept is now supported by NMR results [76, 77] and by studies using fluorescent fatty acids [84].

As far as investigated, fatty acyl-CoA and acyl-L-carnitine esters are bound by each FABP type with an affinity comparable to that of their corresponding fatty acids (apparent K_d 0.1–1 μM) [10–12]. Knudsen [64] noted that data on acyl-CoA binding by purified FABP preparations should be interpreted with caution as they may reflect possible contamination with ACBP. However, this notion may apply only to FABP from tissues which contain amounts of FABP and ACBP of comparable magnitude, such as rat and bovine liver, but not for e.g. rat heart in which the content of FABP is 50 times as high as that of ACBP (Table 1).

The issue of the stoichiometry of fatty acid binding to FABP [12] is now close to being settled. With the NMR technique described above, in which binding can be monitored without perturbation of equilibrium conditions, recombinant rat I-FABP was found to contain a single, high-affinity fatty acid-binding site, rat L-FABP two high-affinity and a third low-affinity binding site, and rat H-FABP one binding site with a high and one with a low affinity [78]. Other striking observations from these studies include a marked pH sensitivity of fatty acid binding by L-FABP but not I-FABP [78], and the exchange rates of fatty acids between bind-

ing sites and unbound pools being severalfold higher for I-FABP than for L-FABP (D.P. Cistola, personal communication).

Of the variety of other methods used to characterize ligand binding to pure FABP, quantitative data have been obtained mostly from assays using spin-labeled or fluorescently labeled fatty acids, and those involving partition of fatty acids between FABP and Lipidex 1000 (at 0° C) or multilamellar liposomes [10, 12]. The large variation in reported binding parameters may reflect differences in and shortcomings of the applied analytical techniques, but also failure to adequately quantitate (i.e. by aminoacyl mass determination) [39, 85, 86] or delipidate [41] the protein prior to performing binding assays. Although theoretically the liposomal transfer assay would underestimate the binding stoichiometry due to the presence in the assay of a lipophilic phase competing with FABP for the binding of fatty acids, binding ratios (but not affinities) in good agreement with the NMR data have been reported by some investigators [86, 87].

The well-established Lipidex 1000 assay [88] is quite suitable for the assessment of both the molar ratio and affinity of the binding of fatty acids to FABP, although weak interactions (e.g. binding of the third fatty acid to L-FABP) cannot be detected [69], provided that possible binding of fatty acids and/or protein to the surface of the test-tube is appropriately corrected for [89]. The addition of Triton X-100 to the assay mixture largely prevents the surface binding without affecting binding parameters to a great extent [89].

Both purified bovine and rat liver ACBP bind C_8–C_{16} acyl-CoA esters at a 1 : 1 molar ratio with nearly equal affinity (apparent K_d 0.1–0.3 μM), but do not bind medium or long-chain fatty acids [64]. It should be noted that these binding parameters are similar to those reported for the binding of $\geq C_{16}$ acyl-CoA esters by $FABP_C$ [90, 91]. However, upon gel-filtration of a mixture of bovine ACBP, L-$FABP_C$ and palmitoyl-CoA, the fatty ester eluted in the ACBP peak only [64], indicating their preferential binding by ACBP under these conditions. Unfortunately, the interaction between ACBP and acyl-L-carnitine has not yet been studied.

Self-aggregation of $FABP_C$

The reversible concentration-dependent self-aggregation behaviour of H-FABP, reported by Fournier and co-workers for rat and pig H-FABP [48, 92–95] has so far been confirmed only qualitatively by Jones *et al.* [96]. The latter investigators observed H-FABP (purified from rat heart or mammary gland) to migrate as a single band on SDS/polyacrylamide-gel electrophoresis, but to be present under non-dissociating conditions in an aggregated as well as a monomeric form in a 4 : 1 ratio. Both protein fractions bound fatty acids with similar capacities [96], which is in contrast to reports by Fournier *et al.* [92–94], who found the self-aggregation process to be in competition with the binding of fatty acids or esters, thereby regulating fatty acid metabolism (see below). Jagschies and colleagues [45], who have investigated the possible self-association of bovine pI-4.9 H-FABP by recording CD spectra under experimental conditions similar to those applied by Fournier [92], and by studying the concentration-dependence of the sedimentation coefficient of the protein, concluded that self-aggregation as a regulative mechanism for binding of fatty acids does not apply to H-FABP from this species. However, Fournier interprets their data as also indicating self-aggregation to occur around a concentration of 2 mg/ml (130 μM) FABP (N.C. Fournier, personal communication). Of the lack of evidence for self-aggregation reported by Offner and co-workers for rat [86] and human H-FABP [33], the former can be explained by the fact that these investigators studied protein concentrations up to only 1.8 mg/ml, but the latter remains at variance with the above-mentioned data.

It is of note that cysteine residues do apparently not play a role in the self-aggregation process, as neither rat [25, 97] nor pig [91] H-FABP contain any cysteine, whereas bovine H-FABP contains one cysteine residue per protein molecule [98]. The reported absence [33] and presence [98, 100] of cysteine in human H-FABP is interpreted to indicate the occurrence of FABP isoforms in human heart [5, 33].

Physiological significance of cytoplasmic and plasmalemmal FABPs and the relation with acyl-CoA binding protein

In the 1970's, $FABP_C$ was commonly considered the cytoplasmic intracellular counterpart of serum albumin, envisaged as a merely passive vehicle for poorly soluble fatty acid and fatty ester molecules. The subsequent establishment of the existence of distinct $FABP_C$ types and isoforms, and the presence and independent regulation of two $FABP_C$ types in the same cell, as occurs in intestinal epithelium and, most likely, in kidney, clearly warrant more specific functions to be attributed to each member of the $FABP_C$ family. A critical evaluation of putative functions of the FABPs has been presented by Ockner [7]. The broad protein heterogeneity found for $FABP_C$ appears not to apply for $FABP_{PM}$ or ACBP. Therefore, these latter proteins may serve in each tissue the same and likely more general biological function.

As pointed out before [10, 12] concepts on the physiological role of $FABP_C$ have been delineated from the analyses of its endogenous ligands and *in vitro* binding properties, its modulatory effects on enzyme activities and from apparent relationships between fluctuations of cytoplasmic $FABP_C$ content and changes in lipid metabolism. Although important for the development of working hypotheses, these latter relationships provide indirect evidence and mostly do not give clues for the mechanism of action of FABP. Another limitation is that L-FABP appears responsive to various physiological changes indeed, yet the other types seem so to a much lesser extent. Extensive surveys of relevant investigations have been presented by others [12, 15]. With respect to more recent developments the elegant studies of Bass and colleagues on the expression of L-FABP in liver in relation to the zonation of fatty acid metabolism should be mentioned [55, 56]. A major finding was that the hepatic L-FABP content of hepatocytes does not govern, but rather is adapted to the cellular flux of fatty acids.

Cistola and colleagues [77] have recently speculated on separate roles played by I- and L-$FABP_C$ in the enterocyte. On the basis of comparative NMR binding studies of the two proteins, these authors have suggested that I-FABP may function to bind and transport fatty acids, i.e. serve as an intracellular albumin in the enterocyte, while the binding specificity of L-FABP could include additional compounds, such as monoacylglycerols, lysophospholipids and bile acids. In this sense L-FABP would serve a function that is analogous to the non-specific binding sites of serum albumin.

Most of the various functions assigned to any of the FABPs (Fig. 1) are still not known with certainty. In addition, the mode of action of the FABPs also remains largely hypothetical, with the exception of the mechanism of the involvement of H-$FABP_C$ in the mitochondrial β-oxidation of long-chain fatty acids, as put forward by Fournier *et al.* on the basis of electron spin resonance studies on isolated cardiac mitochondria [94, 95]. H-FABP was reported to strongly regulate this catabolic system by the process of self-aggregation, with two of the multi-self-aggregated FABP molecular species, namely those existing at about 70 and 140 μM FABP, acting as specific translocators delivering acyl-L-carnitine to the mitochondrial β-oxidative system. Heart energy production thus seems dependent on optimized local H-$FABP_C$ concentrations. Whether this elegant mechanism also applies to other $FABP_C$ types, e.g. that from liver which cells also have a high capacity for mitochondrial fatty acid β-oxidation [101], has not yet been elucidated.

Another fascinating observation on the regulation of FABP is the discovery that a phosphorylated form of aP2 protein (adipocyte FABP) plays an essential role in insulin signal-transduction [5, 21, 102]. This finding warrants investigations into the possible phosphorylation of other FABP types and its conceivably regulatory influence on FABP function. In addition, NMR studies have shown that small changes in intracellular pH might also regulate the binding activity of L-$FABP_C$ [77, 78]. In this context it should be mentioned that the isoforms of $FABP_C$, as found in some tissues, may represent covalently modified and regulated FABP molecules.

$FABP_C$ has also been postulated to serve to protect the cell against undesired effects of long-chain fatty acids, while still having these substrates read-

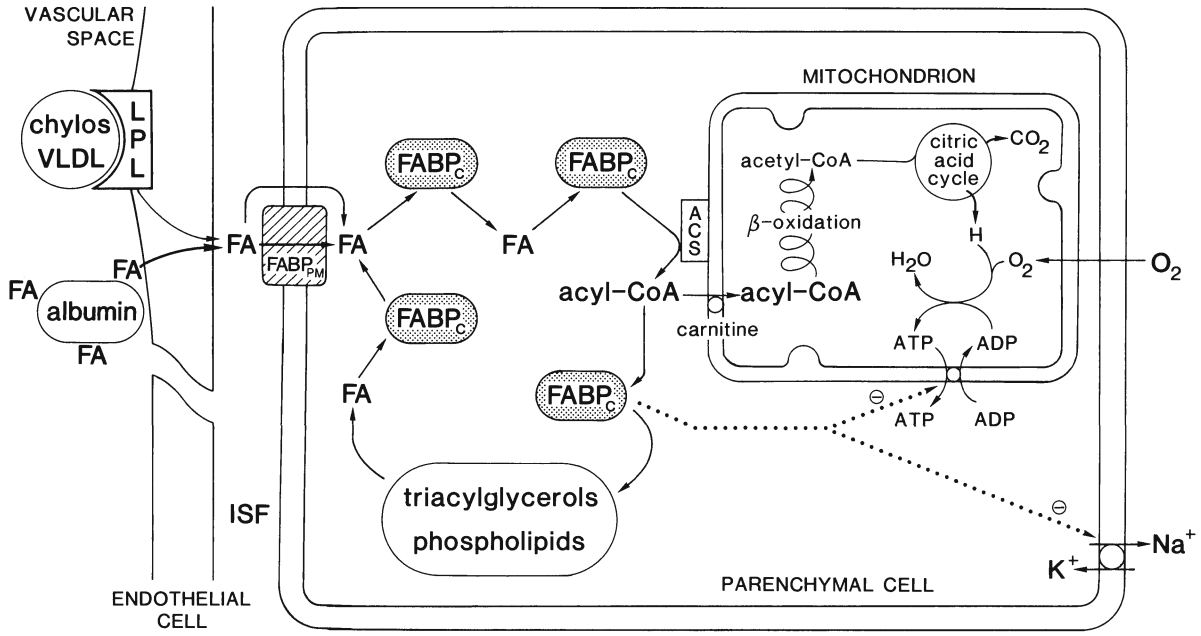

Fig. 1. Schematic presentation of the putative roles of plasmalemmal and cytoplasmic fatty acid-binding proteins ($FABP_{PM}$ and $FABP_C$, respectively) in cellular metabolism of long-chain fatty acids (FA). $FABP_{PM}$ may facilitate the trapping and/or plasmalemmal translocation of fatty acids, and $FABP_C$ the transcytoplasmic movement of poorly-soluble fatty acids and fatty acyl-CoAs from their sites of entry or synthesis to their sites of esterification or oxidation. $FABP_C$ may also modulate (dotted line) effects of these compounds on enzyme activities, such as mitochondrial adenine nucleotide translocase and plasmalemmal Na-K ATPase. VLDL, very-low density lipoproteins; chylos, chylomicrons; LPL, lipoprotein lipase; ACS, acylcoenzyme A synthetase; ISF, interstitial fluid.

ily available to respond to the cell's metabolic needs. This buffering function would be of special importance in heart muscle during prolonged ischemia, when a marked accumulation of fatty acids occurs [60]. Similarly, the suggested role for FABP as free radical scavenger [103, 104] may be of interest.

With respect to the putative role of $FABP_C$ in the transcytoplasmic transport of fatty acyl moieties, e.g. from the plasma membrane to the sites of esterification or oxidation (Fig. 1), some comments should be made. With no FABP present the rate of long-chain fatty acid diffusion directly through the aqueous cytoplasm would be extremely low due to their nearly insolubility as anions in aqueous buffers [105]. FABP could greatly increase the diffusion rate of the fatty acyl moieties, but acting of FABP in a shuttle system, i.e. translocation of the entire FABP-fatty acid complex through the aqueous cytoplasmic compartment, is physiologically not likely to occur. One reason may be that the diffusion of proteins in the cell is limited since most of the native proteins in cytoplasm are associated to the cytoplasmic matrix, rather than being freely dissolved in the aqueous phase [106]. We have earlier suggested [60] that FABP presumably acts by enlarging the effective solubility of fatty acids, thereby diminishing the cytoplasmic diffusion barrier. Transcytoplasmic movement of fatty acyl moieties may then be depicted as passing on of ligands from one FABP molecule to the other. The general abundance of FABP and its relatively low affinity for fatty acids and their esters (as compared with albumin) are in support of this 'stepping-stone' model. The severalfold higher exchange rates observed for oleic acid binding by I-FABP when compared with L-FABP suggests such a function to fit rather I-FABP than L-FABP. However, in relation to its observed binding diversity, L-FABP perhaps serves in the translocation of other ligands for which it may show higher exchange rates [77].

$FABP_c$ and acyl-CoA binding protein

The simultaneous presence in the cytoplasm of two small proteins each exhibiting affinity for the binding of (long-chain) fatty acyl-CoA esters, ACBP and $FABP_C$, raises the issue of the physiological significance of this finding (cf. ref. 64). The overall ACBP content of rat liver (28 nmol/g ww) is about 5-fold lower than that of L-$FABP_C$ (Table 1), but still sufficient to accomodate the reported acyl-CoA content of 16–35 nmol/g ww [107]. This statement also applies to bovine liver, provided that its acyl-CoA content is of comparable magnitude to that of rat liver. However, in rat heart the ACBP content of 2 nmol/g ww provides only a few binding sites for the 15–35 nmol/g ww acyl-CoA esters [60], suggesting the majority of these esters still to be bound by the excessively available H-$FABP_C$. In bovine heart the sum of the contents of $FABP_C$ and ACBP appears relatively low. Although the precise subcellular localization of these proteins and their ligands is not known in detail, this theoretical quantitative comparison indicates striking differences between liver and heart, for which no satisfactory explanation can yet be provided.

Of special interest is the possible interaction of $FABP_C$ and ACBP in the first steps of mitochondrial fatty acid β-oxidation. Of the subsequent intermediates fatty acid, acyl-CoA and acyl-L-carnitine, only acyl-CoA is thought to be bound by ACBP, whereas all three are potentially bound by $FABP_C$. Keeping in mind that liver and heart have a fatty acid-oxidation capacity of similar magnitude [101], the above-mentioned differences in tissue content of the two proteins creates another unresolved issue.

Role of $FABP_{PM}$ in cellular uptake of fatty acids

Fatty acid extraction by e.g. heart muscle or liver is very rapid and remarkably efficient, but the molecular mechanism of their translocation across the various diffusion barriers between the plasma and intracellular compartments is still a controversial subject [108]. Work from the laboratories of Berk [65, 66] and Stremmel [67] suggest that the 40 kDa $FABP_{PM}$ found in the plasmalemmal membrane of many cell types is involved in the transmembrane transport of long-chain fatty acids. Careful analysis of the influx kinetics of oleate into hepatocytes, adipocytes, cardiac myocytes or intestinal epithelial cells have shown that in each case oleate uptake is a saturable function of unbound oleate concentrations. Furthermore, with monospecific antibodies raised against isolated $FABP_{PM}$, specific membrane binding as well as cellular influx of oleate could be selectively inhibited. However, with rat hepatocytes and adipocytes, the influx was inhibited to maximally 50–70% [109, 110], suggesting that only a portion of overall fatty acid uptake is mediated by $FABP_{PM}$.

Indeed, Kammermeier and co-workers [111] have recently provided further evidence for the concept, originally proposed by DeGrella and Light [112], that fatty acids are taken up into the cell by a simple diffusion process. Their studies on palmitate uptake by isolated rat cardiomyocytes indicate fatty acid transfer across the sarcolemmal membrane to be determined by their physicochemical partition between extracellular albumin, the membrane lipid phase, intracellular $FABP_C$ and the respective aqueous phases [111]. The same conclusion was unequivocally reached in studies performed by Noy, Zakim and associates [113–115], in which fatty acid uptake by rat liver was examined by several different experimental approaches. However, passive transport would require the rate of dissociation of fatty acid from its albumin complex to be fast, which is not the case in free solution [116]. Therefore, taken together, these findings may suggest that $FABP_{PM}$ mainly functions in trapping of fatty acids, and that their subsequent transmembrane translocation can occur in two manners, either passively or carrier mediated. The physiological significance of a possible simultaneous existence of these two uptake mechanisms is that it would present additional sites for the efficient regulation of fatty acid metabolism.

Unlike other tissues, in the heart the endothelial cells lining the vascular compartment form a closed layer and hence present an additional barrier for the uptake of fatty acids by the cardiomyocytes. The size of albumin excludes diffusion of the albu-

min-fatty acid complex through interendothelial clefts in seizable amounts, so that fatty acids must be transported across endothelial cells [108]. It would therefore be of interest to examine the possible presence of $FABP_{PM}$ in the (ab)luminal membrane of the vascular endothelial cell.

Conclusions and perspectives

Twenty years of research on cellular fatty acid-binding proteins have yielded suffidient data to indisputably infer their importance in cellular lipid homeostasis, despite the fact that for most assumed functions no definite proof has yet been given. The existence of various distinct types of $FABP_C$ and their type-specific relative abundance in certain tissues remain remarkable and unique features, of which the precise physiological significance still awaits clarification.

Currently available evidence suggests that each of the $FABP_C$ types has adapted to a specific function based on the metabolic needs of the cell type it is expressed in. Such adaptation probably is governed by tissue-specific differences in the subcellular targeting of lipophilic ligands and their subsequent interaction with or protection from (sub) cellular membranes or enzyme systems.

The three classes of lipid binding proteins discussed here, $FABP_C$, $FABP_{PM}$ and ACBP, have in common that each was unexpectedly found to bear a close structural relationship or even to be identical to a protein previously considered as unrelated. Thus, L-$FABP_C$ appeared identical to the mitosis-associated (p14) protein [117], H-$FABP_C$ has a 95% amino acid sequence homology with mammary-derived growth inhibitor [29], K-$FABP_C$ shows a close similarity with urine α_{2U}-globulin [118], $FABP_{PM}$ is closely related to mitochondrial glutamic-oxaloacetic transaminase [66, 67], and ACBP is identical to diazepam-binding inhibitor protein [64]. The functional implications of these recent findings are not yet understood, but will be an important topic to address in the future. Are the $FABP_C$'s directly or indirectly involved in signal transduction and/or growth regulation? It is speculated that $FABP_C$ may deliver its ligand to nuclear *trans*-acting proteins and thereby modulate genes that are coding for key proteins in lipid metabolism or differentiation [5]. Is ACBP principally a neurotransmitter, or is it a protein with a dual function (cf. ref. 64)?

Analysis of cDNA probes of the FABPs will help to unravel the actual number of distinct mammalian FABP types and define the structural relationship among them. Advanced molecular biology techniques, such as site-directed mutagenesis, will also enable the controlled modulation of the expression of the cellular FABPs and ACBP, or of mutants thereof, in e.g. cell cultures under various conditions or transgenic animals [119], so as to more directly permit the delineation of the function of these proteins from the consequence of such manipulation for cell functioning. Expression of functionally active mammalian FABPs in yeast [120] and examination of its effect on lipid metabolic properties of this unicellular eukaryotic microorganism might also be of interest.

The precise determination of the various proteins, types and isoforms will be an important prerequisite for the proper assignment of functions. Because of their, in some cases, very close structural homology, synthetic peptides may be helpful tools for the development of specific immunochemical assays [121].

Finally, attention should be given to the interplay among $FABP_C$, $FABP_{PM}$ and ACBP, both mutually and in interaction with various membrane structures (cf. refs. 122 and 123). The possible coordinate regulation of these proteins could give insight into the participation of each of them in supplying substrates to lipid metabolic pathways.

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