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Studies on fatty acid-binding proteins

The binding properties of rat liver fatty acid-binding protein

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1. The fluorescent fatty acid probe 11-(dansylamino)undecanoic acid binds to rat liver fatty acid-binding protein with a 1:1 stoichiometry. 2. The binding of the fluorescent probe is competitive with long-chain fatty acids. 3. Binding displacement studies were performed with a wide range of fatty acids and other ligands and identified C_{16} and C_{18} fatty acids as the preferred fatty acids for rat liver fatty acid-binding protein. No preference was observed for unsaturated fatty acids within this group. 4. Fatty acyl-CoA binds less well than the corresponding fatty acid.

INTRODUCTION

Fatty acid-binding protein (FABP) from rat liver cytosol is an abundant 14000-M_r monomeric protein that is known to have a high affinity for long-chain fatty acids and their CoA esters, as well as a number of non-polar organic anions (Glatz & Veerkamp, 1985). The precise physiological role of this protein remains unclear, and clarification of its function will require more precise information on its ligand-binding properties, the number of ligand-binding sites and the affinity of the protein for the various types of ligand. Thus, although it is now generally accepted that the protein is involved primarily in fatty acid metabolism, the relative affinity of FABP for free fatty acids and fatty acyl-CoA esters remains controversial (Glatz & Veerkamp, 1985). In addition, the physiological ligand with the highest affinity is haem, although whether *in vivo* it has a role in haem transport is unclear (Vincent & Muller-Eberhard, 1985).

We have previously reported that the fluorescent fatty acid probe 11-(dansylamino)undecanoic acid binds to rat liver FABP with high affinity and shows a considerable enhancement of about 60-fold when compared with the fluorescence of the probe in buffer (Wilkinson & Wilton, 1986). This probe provides a potentially superior method for studying ligand binding than methods relying on the physical separation of bound and free ligands because the fluorescence enhancement allows an essentially instantaneous measurement of the bound ligand. As a result, we have demonstrated that this probe provides an effective method for detecting and quantifying rat liver FABP in biological samples (Wilkinson & Wilton, 1986, 1987*a*; Sheridan *et al.*, 1987). In the present paper, we have extended the work to determine the stoichiometry of binding of 11-(dansylamino)undecanoic acid to rat liver FABP and to assess the binding of other ligands by competitive displacement of the fluorescent probe from the protein. A preliminary report of certain aspects of this work has appeared (Wilkinson & Wilton, 1987*b*).

MATERIALS AND METHODS

Chemicals

11-(Dansylamino)undecanoic acid was obtained from Molecular Probes, Junction City, OR, U.S.A. All ligands used in displacement studies were the highest purity available and were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Rat liver FABP

Rat liver FABP was purified from female Wistar albino rats by the method previously described (Wilkinson & Wilton, 1986).

Fluorescence measurements

All titrations were performed as previously described (Wilkinson & Wilton, 1986). Briefly, the fluorescent ligand was added in small portions (2–10 μl) with a glass microsyringe to a 1 ml assay solution containing buffer (50 mM-potassium phosphate, pH 7.2), protein and additional ligand, as appropriate. The total addition did not exceed 0.05 ml and fluorescence measurements were corrected for dilution. All non-polar ligands were freshly prepared as a 0.1 mM solution in methanol from a stock solution (10 mM) in methanol. A correction had to be made for the effect of methanol on the binding of 11-(dansylamino)undecanoic acid to FABP and on the fluorescence yield of the probe in buffer alone. For some very non-polar ligands, these were also added as 0.1 ml of a 0.01 mM solution in methanol with the appropriate corrections. Fatty acyl-CoA solutions were diluted to 0.1 mM solution in phosphate buffer from a stock solution (10 mM) held at pH 6. Haem was dissolved in 0.2 M-ammonia and carefully diluted to 0.1 mM in phosphate buffer. The binding data for Scatchard (1949) plots were analysed with the LIGAND program of Munson & Rodbard (1980) on an Apple II computer.

Abbreviation used: FABP, fatty acid-binding protein.

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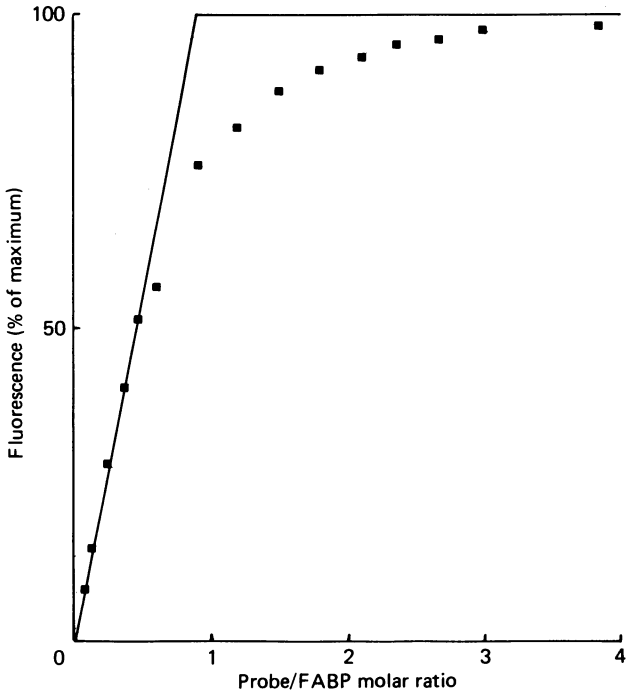

Fig. 1. Binding stoichiometry of 11-(dansylamino)undecanoic acid to rat liver FABP

A titration was performed of 11-(dansylamino)undecanoic acid (0–6 μM) into rat liver FABP (1.60 μM). Fluorescence values are plotted as a percentage of maximum fluorescence.

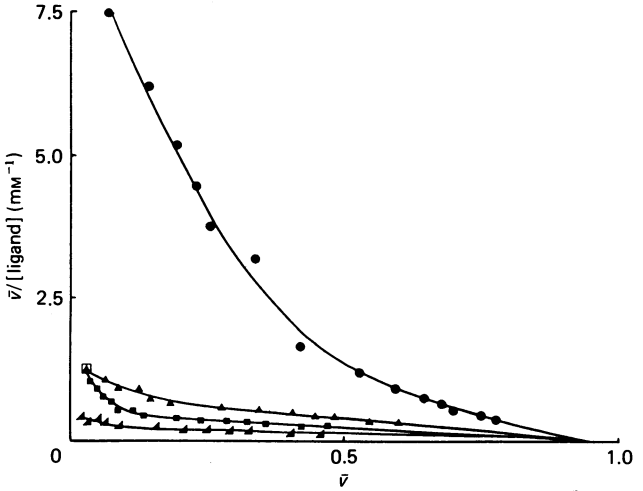

Fig. 2. Scatchard plots of the binding of 11-(dansylamino)-undecanoic acid to rat liver FABP in the absence and in the presence of inhibitors

11-(Dansylamino)undecanoic acid (0.02–5 μM) was titrated into rat liver FABP (0.14 μM) in the absence of inhibitor (●) or in the presence of 1 μM-palmitoyl-CoA (▲), 1 μM-palmitic acid (■) or 1 μM-oleic acid (▲). In this experiment, palmitic acid and oleic acid were added in 10% methanol, giving a final methanol concentration in the assay of 0.1%. $\bar{v}$ is mol of 11-(dansylamino)undecanoic acid bound/total mol of FABP.

RESULTS AND DISCUSSION

Stoichiometry of binding of 11-(dansylamino)undecanoic acid to rat liver FABP

We have reported previously (Wilkinson & Wilton, 1987) a 1:1 stoichiometry of binding of 11-(dansylamino)undecanoic acid to rat liver FABP. This result was obtained by using Scatchard analysis of the binding data, a process that required quantification of the fluorescent yield from protein-bound probe. In view of the disagreement in the literature concerning the stoichiometry of binding of fatty acids to this protein (Glatz & Veerkamp, 1985; Bass, 1985; Offner *et al.*, 1986; Lowe *et al.*, 1987), we have used an alternative approach to determine the stoichiometry of binding of the fluorescent probe. We have plotted fluorescence titration curves in terms of the percentage of maximum fluorescence achieved on saturation of the protein against the molar ratio of probe to protein, as shown in Fig. 1. It is seen that the initial linear part of this curve extrapolates to a 1:1 binding stoichiometry. A similar method has been used to quantify haem binding to rat liver FABP (Tipping *et al.*, 1976; Vincent & Muller-Eberhard, 1985), and again a 1:1 binding was observed.

Although it had previously been accepted that rat liver FABP binds fatty acids with a 1:1 stoichiometry, recent reports have provided evidence that this protein binds 2 mol of fatty acid/mol of protein (Offner *et al.*, 1986; Lowe *et al.*, 1987). This 2:1 stoichiometry is consistent with that reported for the bovine liver FABP (Hauner-land *et al.*, 1984), where it was proposed that the two fatty acid molecules bind at a single site in an anti-parallel fashion (Keuper *et al.*, 1985). However, this bovine FABP binds the bulky fluorescent ligand 16-(9-anthroyl-oxy)palmitic acid with a 1:1 stoichiometry. It would thus appear that liver FABP contains a relatively large binding site able to accommodate a single bulky non-polar anion such as haem or fluorescent fatty acids but able to accommodate two molecules of straight-chain fatty acids.

Displacement of bound 11-(dansylamino)undecanoic acid by fatty acids and other ligands

The specificity of FABP for fatty acids has been the subject of considerable interest, particularly since it may reflect the physiological role of this protein in fatty acid metabolism. We have therefore undertaken an evaluation of fatty acid binding by comparing the ability of different fatty acids and other ligands to displace bound 11-(dansylamino)undecanoic acid with the subsequent loss of fluorescence.

Scatchard (1949) analysis of binding of 11-(dansyl-amino)undecanoic acid to rat liver FABP and the effect of two physiologically important ligands for FABP, palmitic acid and oleic acid, and also palmitoyl-CoA, is shown in Fig. 2. The curved nature of the Scatchard plot for the binding of the fluorescent probe has been observed previously (Wilkinson & Wilton, 1987*b*), and the molecular basis of the apparent heterogeneity of binding requires further investigation. However, the effect of added fatty acid or fatty acyl-CoA on the Scatchard binding curves clearly demonstrates the competitive nature of the ligand binding to FABP.

We therefore decided to investigate the ligand-binding properties of rat liver FABP by determining the effectiveness of each ligand in displacing bound fluorescent probe. The very insoluble nature of long-chain fatty acids, together with the apparent heterogeneity of

Table 1. Displacement of 11-(dansylamino)undecanoic acid from rat liver FABP by fatty acids

To pure rat liver FABP (0.1–0.2 nmol) in 1 ml of buffer was added 0.01 ml of 0.1 mM-11-(dansylamino)undecanoic acid followed by 0.01 ml of 0.1 mM ligand in methanol. The fall in fluorescence due to displacement of the probe from FABP is expressed as a percentage of the initial protein-bound fluorescence. All values are the average for at least three independent determinations involving fresh solutions of ligands. Abbreviation: N.D., not detectable.

Ligand	Displacement (%)
Octanoic acid ($C_{8:0}$)	2.7
Decanoic acid ($C_{10:0}$)	9.7
Lauric acid ($C_{12:0}$)	12.6
Myristic acid ($C_{14:0}$)	16.9
Palmitic acid ($C_{16:0}$)	69.5 (67.0*)
Palmitoleic acid ($C_{16:1}$)	36.3
Stearic acid ($C_{18:0}$)	25.5 (74.5*)
Oleic acid ($C_{18:1}$)	69.6
Linoleic acid ($C_{18:2}$)	49.8
Linolenic acid ($C_{18:3}$)	41.2
Arachidic acid ($C_{20:0}$)	26.0*
Arachidonic acid ($C_{20:4}$)	60.3
Behenic acid ($C_{22:0}$)	23.0*
Docosahexaenoic acid ($C_{22:6}$)	55.0
Methyl palmitate	N.D.*
Oleoyl alcohol	N.D.*
Oleylamine	N.D.*

* For very non-polar ligands these were also added as 0.1 ml of 0.01 mM solution in methanol.

binding of the fluorescent probe to rat liver FABP, meant that the determination of dissociation constants for the various ligands was not possible. However, a ranking order of affinity can be achieved by determining the percentage displacement of fluorescent probe with a 1:1 molar ratio of ligand to probe. Great care was taken to ensure the solubility of the competing ligand, and methanol concentrations of up to 10% (v/v) were employed for certain ligands; however, routinely 1% (v/v) methanol was used with appropriate corrections for the effect of the solvent on ligand binding. Although solvent concentrations of the order of 0.5% have been employed by other workers, in our hands these lower concentrations of solvent gave variable results with long-chain fatty acids and, in particular, enhanced the apparent effectiveness of binding of unsaturated fatty acids relative to saturated fatty acids (see Fig. 2). The problems of assays involving long-chain fatty acids and knowing the true concentration of monomeric anion in solution cannot be overemphasized.

These results are summarized in Table 1. Saturated fatty acids shorter than C_{16} bind only poorly to rat liver FABP, whereas with saturated fatty acids longer than C_{18} it would appear that binding is decreased. Where a saturated fatty acid is readily accommodated by FABP, the corresponding unsaturated fatty acid is a less effective displacer of the fluorescent probe. This effect is clearly shown by the C_{18} fatty acid series, where the insertion of each double bond causes the apparent affinity of the fatty acid for FABP to be diminished. For fatty acids longer than C_{18} additional double bonds appear to enhance binding. The removal of the negatively charged carboxy group, as with methyl palmitate, oleoyl alcohol and oleylamine, results in no measurable displacement of the fluorescent probe, confirming the apparent requirement for the ligands of FABP to be anionic.

Table 2. Displacement of 11-(dansylamino)undecanoic acid from rat liver FABP by various ligands

Assay conditions are as described in Table 1. Abbreviation: N.D., not detectable.

Ligand	Displacement (%)
Haem	> 95
Palmitoyl-CoA	23.5
CoA	N.D.
Palmitoylcarnitine	N.D.
Malonyl-CoA	N.D.
Cholesterol sulphate	10.0
Cholesterol	N.D.*
Prostaglandin E_1	N.D.

* For this very non-polar ligand, it was also added as 0.1 ml of 0.01 mM solution in methanol.

The above results are consistent with a hydrophobic binding site on FABP of defined size and containing a positively charged group as the counterion for the negatively charged carboxy group. Much of the binding interaction can be explained by the hydrophobic effect, which will increase with the length of the alkyl side chain of the fatty acid. However, the results suggest that, with alkyl chain length longer than C_{18}, this increasing interaction is offset by a diminished ability of the site to accommodate the alkyl side chain effectively. The introduction of double bonds into the alkyl chains decreases the hydrophobicity of the ligand (Tanford, 1973). However, it will also make the alkyl chain shorter and more bulky (Brenner, 1984), and may explain why the two physiologically important polyunsaturated fatty acids arachidonic acid ($C_{20:4}$) and docosahexaenoic acid ($C_{22:6}$) are apparently more readily accommodated than arachidic acid ($C_{20:0}$) and behenic acid ($C_{22:0}$) respectively.

The ability of a number of other physiologically important compounds to displace bound 11-(dansylamino)undecanoic acid from rat liver FABP was also investigated, and the results are shown in Table 2. Long-chain fatty acyl-CoA is less readily able to bind to FABP than the corresponding free fatty acid, an observation that is consistent with the work of Bass (1985). Since the acyl-CoA lacks the carboxylate group of the parent free acid, presumably the CoA provides a suitable negative charge resulting from an appropriate folded conformation of the palmitoyl-CoA. Oleoyl-CoA behaved in an essentially identical manner (results not shown). Haem was found to be the most effective ligand tested with this displacement method, and this confirms the observations of other workers (Vincent & Muller-Eberhard, 1985; Bass, 1985).

Both cholesterol sulphate and oestrone sulphate (results not shown) were moderately effective ligands for this protein, consistent with its original identification as an anion-binding protein (Levi *et al.*, 1969; Ketterer *et al.*, 1976). However, we have been unable to detect binding of cholesterol by this method. Hence the cholesterol-binding site that has been reported by some

workers for rat liver FABP (Schroeder *et al.*, 1985) must be separate from the fatty acid-binding site. We were unable to confirm the report that prostaglandin E_1 is able to bind to FABP (Dutta-Roy *et al.*, 1987).

General discussion

There is a considerable variation in the literature as to the stoichiometry, specificity and affinity of ligands for FABP. These differences probably reflect the difficulty of performing binding studies with some of the highly insoluble compounds that are the natural ligands for this protein. The fluorescence-enhancement technique employed in the present study has the advantage of providing an essentially instantaneous measure of bound ligand, and this fluorescent probe is remarkably stable and relatively soluble.

The stoichiometry of binding of ligands has important implications in terms of quantification of FABP in the liver by ligand-binding sutides. The majority of these methods have relied upon the measurement of the amount of protein-bound radioactive fatty acid under saturating conditions and have assumed a 1:1 stoichiometry of binding to calculate mol of FABP/mol. These calculations have produced average values of between 4 and 6% for the percentage of cytosolic protein that is FABP (Glatz *et al.*, 1984; Paulussen *et al.*, 1986). Using the fluorescent-enhancement method, we have reported values over the range 2–3% (Wilkinson & Wilton, 1986). If the correct stoichiometry of binding for normal fatty acids such as palmitate and oleate is in fact 2:1, this will necessitate a correction for these rat liver FABP assays involving radioactive fatty acids and will lower these values to between 2 and 3% for the percentage of cytosolic protein that is FABP.

Using the method of competitive displacement at a 1:1 ratio of fluorescent probe to ligand we have confirmed the preference of rat liver FABP for long-chain fatty acids (C_{16} and C_{18}). However, we have not observed the reported preference of this protein for long-chain unsaturated fatty acids provided that sufficient solvent is included in the assay to ensure the solubility of the ligand. It should be noted that any assay conditions that are not optimal for keeping long-chain fatty acids in solution will tend to favour the solubility and effectiveness of unsaturated fatty acid relative to the corresponding saturated fatty acid.

In view of the apparent heterogeneous binding of 11-(dansylamino)undecanoic acid to FABP, we have not attempted to quantify the actual affinity of fatty acids or other ligands for this protein. However, it is presumed that fatty acids such as palmitate and oleate must be binding with a higher affinity than the fluorescent probe to achieve the greater than 50% displacement at equimolar ratios.

The binding of the fluorescent fatty acid probe to rat liver FABP can be resolved into two components with apparent dissociation constants of about 0.03 μM and 0.5 μM (Wilkinson & Wilton, 1987*b*). Combining this information with the displacement studies described in the present paper indicates dissociation constants for palmitic acid and oleic acid considerably lower than those reported in the literature from radioactive-ligand-binding studies. Values of up to 3 μM have been reported for these fatty acids binding to rat liver FABP (Lowe *et al.*, 1987). This discrepancy between the two methods and the apparent heterogeneity of binding of the fluorescent probe to FABP require further investigation, but may reflect the problem of ligand solubility and, hence, the determination of the real concentration of free monomeric anion in the binding assay.

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