Studies on fatty acid-binding proteins

The detection and quantification of the protein from rat liver by using a fluorescent fatty acid analogue

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Fatty acid-binding protein from rat liver is shown to bind the fluorescent fatty acid probe dansyl undecanoic acid. Binding is accompanied by a shift in the fluorescence emission maximum from 550 nm to 500 nm and a 60-fold fluorescence enhancement at 500 nm. These spectral properties have allowed the use of this probe to detect and quantify μg amounts of liver fatty acid-binding protein during purification procedures. In conjunction with h.p.l.c. the method allows the rapid estimation of liver fatty acid-binding protein in biological samples. The validity of the method is demonstrated by measuring the concentration of fatty acid-binding protein in livers from control and hypolipidaemic-drug-treated rats. The dramatic diurnal rhythm previously reported for this protein [Dempsey (1984) *Curr. Top. Cell. Regul.* **24**, 63–86] was not observed with this method.

INTRODUCTION

Fatty acid-binding protein (FABP) from rat liver is an M_r 14000 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). Although its precise function *in vivo* has not yet been defined, it may be involved in the intracellular transport and compartmentation of long chain fatty acids, or it may protect specific enzymes against inhibition by long chain acyl-CoA esters (Glatz & Veerkamp, 1985; Grinstead *et al.*, 1983). Support for the first of these hypotheses comes from the observation that hepatic FABP concentration is altered by conditions that affect the uptake and utilization of free fatty acids (Fleischner *et al.*, 1975; Ockner *et al.*, 1979; Renaud *et al.*, 1978).

Most early methods for the detection and estimation of this protein in cytosol have relied upon its ligand-binding properties and, in particular, on the co-elution of radioactive fatty acids from a gel filtration column. This procedure, however, is not very sensitive because of ligand–matrix interactions and the possibility of protein–protein competition for the ligand (Morrow & Martin, 1983). In addition, the fractions obtained are radioactive and subsequent studies on the protein are more difficult to perform. More recently, a radiochemical assay procedure based on the use of Lipidex 1000 to separate free from protein-bound labelled fatty acids has become the preferred method for estimating FABP concentrations in liver cytosols (Glatz & Veerkamp, 1983; Glatz *et al.*, 1984).

In this paper we describe a new technique for the detection and estimation of FABP which makes use of the fluorescence enhancement observed when a fluorescent fatty acid probe binds to FABP. The probe we have used is dansyl undecanoic acid, because not only does it bind to FABP with high affinity, but it also showed

considerable fluorescence enhancement. These properties have allowed us to detect FABP down to levels of $1 \mu\text{g/ml}$.

The fluorescent probe has been used for the rapid detection of FABP during purification procedures, and we also describe the use of the probe, in conjunction with high performance gel permeation chromatography, for the rapid estimation of FABP in cytosols prepared from rat liver. The validity of this technique has been confirmed by analysis of liver cytosols from rats that had been fed diets containing the hypolipidaemic drugs tiadenol and clofibric acid, which are known to enhance the levels of hepatic FABP (Kawashima *et al.*, 1983; Bass *et al.*, 1985a; McTigue *et al.*, 1985). Finally, we have used this technique to examine the diurnal variation in hepatic FABP content which has been reported by some workers (Glatz *et al.*, 1984; Dempsey, 1984).

MATERIALS AND METHODS

Chemicals

Dansyl undecanoic acid was obtained from Molecular Probes, Junction City, OR, U.S.A. Sephadex G-75 was obtained from Pharmacia and DE-52 was obtained from Whatman. Clofibric acid was obtained from Sigma and tiadenol was obtained from Aldrich. All other chemicals used in this study were of analytical grade quality.

Animals

Female Wistar Albino rats weighing 200 g were used throughout this study. Rats were fed *ad libitum* on a commercial diet. Rats of drug-treated groups were fed *ad libitum* for 7 days before death on a diet containing either 0.5% clofibric acid or 0.5% tiadenol. The diets containing drugs were prepared by spraying ordinary pelleted rat chow with an acetone solution of the drug followed by thorough air drying.

Abbreviations used: FABP, fatty acid-binding protein; dansyl, 11-[(5-dimethylaminonaphthalene-1-sulphonyl)amino].

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Rats used for the study of the diurnal variation of FABP were maintained for 1 week before death under conditions of reversed lighting (dark period 04:00 h to 16:00 h).

Preparation of rat liver cytosolic fraction

Rats were killed by cervical dislocation. Livers were isolated and used to prepare a homogenate (1:2.5, w/v) in ice-cold 0.1 M-potassium phosphate buffer (pH 7.4) containing 5 mM-EDTA. The homogenate was centrifuged for 30 min at 10000 *g* and the resulting supernatant was centrifuged for 90 min at 105000 *g*. The clear supernatant obtained was aspirated to remove contaminating floating fat to give the cytosolic fraction.

Purification of fatty acid-binding protein

Cytosol was prepared from four 200 g rats as described above. Further purification was based on the methods of Dempsey *et al.* (1981) and Takahashi *et al.* (1983). The supernatant left after precipitation with $(\text{NH}_4)_2\text{SO}_4$ at 70% saturation was dialysed against 10 mM-potassium phosphate buffer (pH 7.4), concentrated by ultrafiltration (Amicon YC05 membrane) and applied to a column of Sephadex G-75 (4.5 cm $\times$ 65 cm) equilibrated with 10 mM-potassium phosphate, pH 7.4. The FABP fraction with binding activity for dansyl undecanoic acid (or [$1\text{-}^{14}\text{C}$]oleic acid in preliminary experiments) was dialysed against 30 mM-Tris/HCl, pH 9.0, concentrated by ultrafiltration (Amicon YC05 membrane) and applied to a column of DEAE-cellulose (Whatman DE-52, 2 cm $\times$ 12 cm) equilibrated with 30 mM-Tris/HCl, pH 9.0. The column was initially eluted with the equilibrating buffer followed by elution of FABP with the same buffer containing 64 mM-NaCl. Homogeneity of the final preparation was confirmed by the appearance of a single protein band on SDS/polyacrylamide-gel electrophoresis (15% acrylamide gels; Laemmli, 1970) and a single peak on analysis by gel-permeation h.p.l.c. (LKB TSK-G 2000 SW column, 7.5 mm $\times$ 600 mm).

H.p.l.c. analysis

H.p.l.c. of FABP and liver cytosols was performed using an LKB 2150 system and a TSK-G 2000 SW gel permeation column (7.5 mm $\times$ 600 mm) equilibrated with 50 mM-potassium phosphate buffer, pH 7.2. The flow rate was set at 1 ml/min, and 1 ml fractions were collected for use in fluorescence measurements.

The h.p.l.c. system was calibrated for the determination of FABP content of cytosolic fractions by subjecting various amount of purified FABP to h.p.l.c. followed by determination of the fluorescence intensity of the appropriate fractions using dansyl undecanoic acid fluorescence enhancement. The data was used to construct a standard curve of fluorescence intensity against the amount of FABP assayed.

Analysis of liver cytosolic fractions in order to quantify FABP content was carried out by subjecting 20 μl of cytosolic fractions (15 mg/ml protein concentration) to h.p.l.c. followed by analysis of aliquots of column fractions using dansyl undecanoic acid.

Fluorescence measurements

Dansyl undecanoic acid was initially dissolved in methanol to give a 1 mM solution. A 0.1 mM stock solution, which was used for fluorescence measurements,

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified FABP

Purified FABP (10 μg , track 1) was subjected to electrophoresis on a 15% SDS/polyacrylamide gel. M_r markers are shown in track 2.

was prepared by slow addition of 50 mM-potassium phosphate buffer, pH 7.2, to avoid precipitation of the probe.

Fluorescence measurements were carried out in a Perkin-Elmer 650 S fluorescence detector. The temperature was controlled at 20 $^{\circ}\text{C}$ by circulating water through the cell holder. To the cuvettes containing 1 ml of buffer or protein sample were added small aliquots (2 μl) of dansyl undecanoic acid stock solution (0.1 mM) by using a microsyringe. Total volume added did not exceed 0.05 ml. Fluorescence intensity was measured at 500 nm after excitation at 350 nm, with a slit width of 5 nm for both excitation and emission. Maximum fluorescence enhancement was accurately determined for aliquots of h.p.l.c. column fractions by inspection of the fluorescence titration data obtained after addition of aliquots of

Fig. 2. Fluorescence emission and excitation spectra of dansyl undecanoic acid

The emission (a) and excitation (b) spectra of dansyl undecanoic acid were recorded in 50 mM-phosphate buffer, pH 7.2 (lower spectra) and in the presence of FABP (upper spectra). Lower spectra were obtained with an instrument sensitivity 10-fold greater than for the upper spectra. Each sample contained $1 \mu\text{M}$ -dansyl undecanoic acid. For the upper spectra, FABP was present at $50 \mu\text{g/ml}$. For (a) excitation was at 350 nm. For (b) emission was at 500 nm.

dansyl undecanoic acid. Alternatively, for rapid detection of FABP in column fractions a single addition ($10 \mu\text{l}$) of a 1 mM solution of dansyl undecanoic acid was used.

Protein determination

Protein was located in chromatographic fractions by measuring A_{280} .

The concentration of protein in cytosolic fractions was determined by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as standard. The concentration of protein in purified FABP preparations was determined by (i) the method of Bradford (1976) with bovine serum albumin as standard, (ii) the biuret method or (iii) the spectrophotometric method of Whitaker & Granum (1980).

RESULTS AND DISCUSSION

FABP was purified from rat liver by $(\text{NH}_4)_2\text{SO}_4$ fractionation, Sephadex G-75 gel filtration and DEAE-cellulose chromatography (Dempsey *et al.*, 1981; Takahashi *et al.*, 1983). The FABP obtained gave a single band on SDS/polyacrylamide-gel electrophoresis as shown in Fig. 1, and had an apparent M_r of 12000 which is consistent with the literature (Ockner *et al.*, 1982). During the purification procedure, FABP in chromatographic fractions was located in preliminary experiments by co-elution of $[1-^{14}\text{C}]$ oleic acid as described by Ockner *et al.* (1982). This technique, however, has the disadvantage of contaminating the FABP preparations with radioactive material, making further studies on the protein more difficult to perform. In view of the successful application of fluorescent fatty acid probes to the study of albumin, we decided to make use of a

fluorescent probe to detect FABP in column fractions during purification procedures in order to obviate this problem.

Dansyl undecanoic acid has been used as a probe to study the properties of lipid bilayers (Rooney *et al.*, 1983) and is known to show a considerable fluorescence enhancement and a blue shift of the emission wavelength maximum when located in a non-polar environment. It also has the advantage over the parinaric acids, which have been used in the study of serum albumin, of being very stable. In Fig. 2, we demonstrate the spectral properties of this probe when measured in phosphate buffer and when bound to FABP. As can be seen, there is a shift of both the excitation and emission wavelength maxima, the emission wavelength maximum being shifted from 550 nm to 500 nm when the probe is bound to protein. This is consistent with the probe binding to a non-polar site on FABP; a similar wavelength shift is observed when the probe is bound to human serum albumin (results not shown).

When fluorescence measurements are made at 500 nm after excitation at 350 nm, the fluorescence enhancement compared with measurement of the same probe in buffer is about 60-fold when all the probe is bound to FABP. This fluorescence enhancement value may be obtained by plotting $1/F$ against $1/[\text{FABP}]$ (where F is fluorescence intensity in arbitrary units) for a fixed concentration of probe from which the maximum fluorescence at infinite FABP concentration may be obtained (Horowitz & Criscimagna, 1985). This enhancement of fluorescence is also characteristic of binding to a non-polar site on the protein. Furthermore, the fluorescent probe appears to bind at the fatty acid-binding site of FABP as palmitic acid and oleic acid cause displacement of the probe,

Fig. 3. Elution profile of 70% $(\text{NH}_4)_2\text{SO}_4$ supernatant of rat liver cytosol from a Sephadex G-75 column

Fractions (9 ml) were collected and 100 μl aliquots were used for fluorescence measurements. Dansyl undecanoic concentration was 10^{-5} M.

which was detected by a concomitant decrease in fluorescence intensity with increasing fatty acid concentration.

This considerable fluorescence enhancement, which could be obtained even at low probe concentrations of 10^{-6} – 10^{-7} M, allowed us to use this probe as a method for detecting the presence of FABP in column fractions during purification procedures. The validity of this approach is clearly illustrated in Fig. 3 which shows the result of the separation of a 70% $(\text{NH}_4)_2\text{SO}_4$ supernatant from rat liver cytosol on Sephadex G-75 using dansyl undecanoic acid fluorescence enhancement to detect the FABP peak. The characteristic low- M_r peak of FABP was clearly shown and, in addition, a further peak was observed at a higher M_r , which was probably due to residual albumin. [The sensitivity of the probe for FABP may be further enhanced if a lower concentration of probe (10^{-6} M) is used.] Fig. 4 shows the elution profile of purified FABP from a DE-52 column, and again the major peak due to FABP is clearly detected by the probe.

The effectiveness of this technique for detecting FABP encouraged us to develop the method to allow the estimation of FABP levels in rat liver cytosols. In order to facilitate the analysis of liver cytosols we decided to make use of gel permeation h.p.l.c. rather than conventional separation on Sephadex G-75 because of the speed and high reproducibility of separations made by this technique. In Fig. 5 we show the result of the separation of 300 μg of liver cytosol on a TSK-G 2000 SW column. Analysis of aliquots of column fractions allowed the rapid detection of the peak corresponding to FABP. This peak corresponded precisely to the retention time of electrophoretically homogeneous FABP when run under identical conditions. This standard FABP was also chemically pure as assessed by comparison of its amino acid composition with data already published (Takahashi *et al.*, 1983).

The validity of this approach to estimate FABP is demonstrated in Fig. 6 in which maximum fluorescence intensity was plotted against the amount of FABP that had been subjected to h.p.l.c. A linear relationship was

Fig. 4. Elution profile of the FABP fraction from DE-52 cellulose

FABP fraction, obtained from the G-75 separation (Fig. 3), was applied to a column of DE-52 equilibrated with 30 mM-Tris/HCl buffer, pH 9. Elution of FABP was carried out with 64 mM-NaCl. Fractions (4.5 ml) were collected and 50 μl aliquots were used for fluorescence measurements.

Fig. 5. H.p.l.c. analysis of rat liver cytosol and location of FABP using dansyl undecanoic acid fluorescence enhancement

(a) H.p.l.c. analysis of rat liver cytosol fraction: 20 μl of cytosol (15 mg of protein/ml) was run on a TSK-G 2000 SW column (7.5 mm $\times$ 600 mm). The position of elution of pure FABP is indicated. (b) Fractions (1 ml) were collected during the h.p.l.c. analysis and 500 μl aliquots of these fractions were used for fluorescence titrations.

Fig. 6. Fluorescence assay of FAPB

Various amounts of pure FAPB were run on a TSK-G 2000 SW gel permeation h.p.l.c. system and the maximum fluorescence intensity obtained due to binding of dansyl undecanoic acid to FAPB was plotted against the amount (μg) of FAPB in the assay cuvette.

obtained, with a lower limit of detection for FAPB of about $1 \mu\text{g/ml}$. The recovery of FAPB after h.p.l.c. was shown to be of the order of 95% by using [^{14}C]FAPB labelled by reductive methylation of the protein according to the procedure of Dottavio-Martin & Ravel (1979) (T. C. I. Wilkinson & D. C. Wilton, unpublished work). This essentially quantitative recovery of FAPB was also shown to be the case after the separation of cytosols by adding known amounts of pure FAPB to liver cytosol followed by estimation by fluorescence measurements of the total FAPB content in column fractions obtained after h.p.l.c.

In order to use this technique to give accurate estimates of FAPB levels in liver cytosols, precise quantification of the pure FAPB is necessary in order to give an accurate standard curve. With purified FAPB preparations, the determination of the protein by the biuret method or by the method of Bradford (1976) appear to be inadequate for this purpose when using bovine serum albumin as a standard (Table 1). The spectrophotometric method of Whitaker & Granum (1980) was found to be much more suitable for the determination of FAPB concentrations of pure samples. This was confirmed by gravimetric analysis of the protein present in the samples used for comparison of the three methods. Thus the spectrophotometric method of Whitaker & Granum (1980) was adopted as the routine method for the determination of the protein concentration of purified FAPB preparations. Similar problems in the quantification of purified liver FAPB by using the method of Lowry have also been encountered in the literature (Glatz *et al.*, 1985), emphasizing the need for caution in applying these methods to the determination of amounts of purified protein when using other proteins as standards.

Having verified that the use of gel permeation h.p.l.c.

Table 1. Comparison of methods for the determination of FAPB concentration in pure preparations

The concentration of pure protein in a preparation of FAPB was assessed by using the three methods described below. Each measurement was performed in duplicate. The actual mass of protein present in the sample was determined gravimetrically and was in close agreement with the value obtained using the method of Whitaker & Granum (1980). The biuret and Bradford methods used serum albumin as the standard protein.

Method	Protein concentration (mg/ml)
Biuret	3.43
Bradford	0.79
Spectrophotometric (Whitaker & Granum, 1980)	1.68

Table 2. Effect of administration of clofibric acid and tiadenol on the level of FAPB in rat liver cytosols

Rats were fed a control diet or a diet containing 0.5% clofibric acid or 0.5% tiadenol for 1 week before death. The FAPB content of rat liver cytosols was determined by subjecting the cytosol to h.p.l.c. followed by analysis of the fractions containing FAPB using dansyl undecanoic acid fluorescence enhancement. Values are mean $\pm$ S.D. Numbers in parentheses are the number of animals used.

Treatment	FAPB content ($\mu\text{g/mg}$ of cytosolic protein)
Control (4)	30.2 ± 4.2
Clofibric acid (4)	51.0 ± 5.3
Tiadenol (4)	78.5 ± 5.8

coupled with the use of the fluorescence enhancement of dansyl undecanoic acid was a valid technique for the estimation of FAPB levels in rat liver cytosols, we were in a position to verify the changes in FAPB levels which have been observed previously under different physiological and pharmacological conditions (Kawashima *et al.*, 1983; Glatz *et al.*, 1984).

The most dramatic changes in FAPB are produced by various hypolipidaemic drugs which produce peroxisomal proliferation, such as clofibrate and tiadenol (Kawashima *et al.*, 1983). We, therefore, subjected rats to a diet containing 0.5% of either of these two drugs for 7 days and then prepared cytosolic fractions from the livers. The cytosols were then subjected to h.p.l.c. as described above and the FAPB peak was quantified by using the fluorescence enhancement method described above. The results are shown in Table 2. It can be seen that both clofibrate and tiadenol cause an increase in cytosolic FAPB levels, and tiadenol was found to be more effective in this respect than clofibrate. This is consistent with the literature available to date (Kawashima *et al.*, 1983).

Having established the validity of the technique with a well-described experimental manipulation we decided to extend our observations to the proposed diurnal variation of FAPB concentration in rat liver, as this area is somewhat more controversial. Thus, Dempsey (1984)

Table 3. Effect of a 12 h light/12 h dark cycle on the level of FABP in rat liver cytosols

Rats were maintained on a cycle of alternating 12 h periods of light and darkness (dark period 04:00–16:00) for 1 week before death at the times stated. Rats were fed *ad libitum* throughout this period. FABP content of rat liver cytosols was determined by subjecting the cytosol to h.p.l.c. followed by analysis for the fraction containing FABP using dansyl undecanoic acid fluorescence enhancement. Values are mean $\pm$ S.D. Numbers in parentheses are the number of animals used.

Time	FABP content (μ g/mg of cytosolic protein)
10:00 h (3)	30.3 $\pm$ 0.57
16:00 h (4)	20.9 $\pm$ 0.8
22:00 h (4)	26.2 $\pm$ 2.9
04:00 h (4)	22.4 $\pm$ 3.5

has reported up to a 10-fold variation in FABP concentration during a 12 h light/12 h dark cycle in rat liver. This contrasts with the results of Bass *et al.* (1985b) who have observed only a modest diurnal variation which was not statistically significant. Table 3 shows the results obtained using our fluorescence enhancement technique. We only observed modest changes in FABP levels in rat liver during an alternating 12 h light/12 h dark cycle, which is more consistent with the results of Bass *et al.* (1985b). The reason for the discrepancy between our results and those of Dempsey (1984) are unknown at this stage, but may be related to the method of quantification of the protein.

A potential criticism of this work might arise from the fact that FABP is known to be associated with non-covalently bound fatty acids which could affect the results obtained using this assay. We have found, however, that when samples of cytosol were delipidated by extraction with di-isopropyl ether according to the method of Ockner *et al.* (1982) and subsequently subjected to h.p.l.c., no difference was observed in the maximum fluorescence enhancement produced by the FABP fractions when compared with non-delipidated samples. This observation is consistent with the fact that the fatty acid probe and endogenous fatty acids both bind reversibly at the same site on FABP and hence addition of excess probe will result in displacement of any bound fatty acids.

The levels of FABP that were observed using the techniques described above are within the range quoted in the literature for rat liver FABP (10–100 μ g/mg of cytosolic protein). This extreme range of reported values may be the result of both biological variation (particularly in terms of the sex and stage of development of the rats used) and the different techniques used to measure this protein.

In summary, we have described a new technique for the detection and assay of FABP which relies on the fluorescence enhancement produced when dansyl undecanoic acid binds to the protein. Using this technique we have confirmed that the hypolipidaemic drugs, clofibrate and tiadenol, produce a marked increase in the concentration of this protein when administered to rats.

We have also demonstrated that there is no major diurnal variation in FABP concentration in rat liver cytosol as assessed using this technique. This is in marked contrast to the results of Dempsey (1984) who has reported a 10-fold diurnal variation in FABP concentration in rat liver. The reason for this discrepancy is unknown, but our results are in agreement with those of Bass *et al.* (1985b) who have also been unable to show a statistically significant change in FABP concentration during an alternating light/dark cycle. In the near future we hope to make use of this assay to delineate further the alterations in FABP levels that might occur under different physiological and pharmacological conditions. We are also using this fluorescent fatty acid probe to investigate the binding properties of purified liver FABP.

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