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Acyl-CoA-binding protein from cow

Binding characteristics and cellular and tissue distribution

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Using the tyrosine fluorescence quenching as a criterion for acyl-CoA binding, we have shown that acyl-CoA-binding protein (ACBP) binds acyl-CoA esters with a chain length $> C_8$ with equal affinity. The binding studies indicated a binding stoichiometry of 1 mol of acyl-CoA/2 mol of ACBP. The protein was found in liver, adipose tissue, intestinal mucosa, kidney, heart, brain, muscles and mammary gland. The highest concentration was found in liver cytosol and the lowest in muscles and mammary gland. ACBP could not be shown to bind non-esterified fatty acids.

INTRODUCTION

We have previously shown that bovine liver contains a novel acyl-CoA-binding protein (ACBP) with the ability to induce medium-chain fatty acid synthesis by goat mammary-gland fatty acid synthetase (Mogensen *et al.*, 1987). ACBP has been purified and its complete amino acid sequence has been determined (Mikkelsen *et al.*, 1987). The amino acid sequence of ACBP did not show any homology with that of fatty-acid-binding protein (FABP). FABP has nearly the same M_r as ACBP and binds both the fatty acids and long-chain acyl-CoA (Glatz & Veerkamp, 1984). In the present paper, we investigate the binding properties of the protein and its intracellular localization and occurrence in different tissues.

MATERIALS AND METHODS

Chemicals

Dansyl-undecanoic acid was a gift from Dr. D. C. Wilton, Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K. Acetyl-CoA was synthesized enzymically as described by Hansen *et al.* (1984). Butyryl-, octyl-, decanoyl-, dodecanoyl-, tetradecanoyl- and hexadecanoyl-CoA were synthesized by the method of Sanchez *et al.* (1973). The concentration of acyl-CoA was determined by using an absorption coefficient of 14500 $M^{-1} \cdot cm^{-1}$ at 260 nm. CNBr-activated Sepharose 4B was from Pharmacia, Uppsala, Sweden. Nitrocellulose (BA 83) was from Schleicher and Schuell, Dassel, Germany. Pre-stained protein M_r standards [lysozyme, M_r 14300; β-lactoglobulin, M_r 18400; α-chymotrypsinogen, M_r 25700; ovalbumin, M_r 43000; bovine serum albumin (BSA), M_r 68000; phosphorylase *b*, M_r 97400; myosin, M_r 200000] were from Bethesda Research Laboratories, Cambridge, U.K. Alkaline-phosphatase-conjugated affinity-purified swine anti-rabbit IgG was from Dakopatts, Glostrup, Denmark. Micro-titre plates were from NUNC, Arhus, Denmark. All other chemicals used in this study were of analytical grade.

Purification of ACBP

ACBP was isolated from cow liver as described by Mikkelsen *et al.* (1987). The protein was stored as freeze-dried powder or in solution in distilled water at −20 °C.

Fluorescence measurements

Fluorescence measurements were carried out on a Spex Fluorolog 2 spectrofluorimeter at 20 °C. To the cuvette containing 1.2 ml of 20 mM-potassium phosphate buffer, pH 7.0, and 10 μM-ACBP were added increasing amounts of different acyl-CoAs or non-esterified fatty acids in small volumes (5–40 μl) of 20 mM-potassium phosphate buffer, pH 7.0. The total volume of added acyl-CoA solution did not exceed 0.14 ml, and the fluorescence values were corrected for the dilution effect. The fluorescence measurements with dansyl-undecanoic acid were carried out as described by Wilkinson & Wilton (1986).

Production and detection of antibody

ACBP was coupled to keyhole-limpet haemocyanin with glutaraldehyde as follows. To 1.5 mg of the haemocyanin in 100 μl of 0.1 M-potassium phosphate buffer, pH 6.8, was added 30 μl of glutaraldehyde at 25 °C. The reaction was allowed to continue overnight at room temperature. Glutaraldehyde that had not reacted was separated from the activated haemocyanin by gel filtration on a short Sephadex G-25 column (3 cm × 0.5 cm) equilibrated with 0.15 M-NaCl. To the activated haemocyanin was added 5.2 μl of 1.0 M-$NaHCO_3$ buffer (pH 9.5)/ml and 200 μg of ACBP, and the reaction was allowed to proceed overnight at 4 °C. Aldehyde groups on haemocyanin that had not reacted were conjugated to a large excess of glycine. The haemocyanin–ACBP complex was dialysed against 20 mM-potassium phosphate buffer (pH 7.4)/150 mM-NaCl (PBS) overnight to remove glycine that had not reacted. The conjugated ACBP–haemocyanin (200 μg) was injected subcutaneously in complete Freund's adjuvant into rabbits. Injections were repeated every 2 weeks with 200 μg of the conjugated ACBP–haemocyanin in incomplete Freund's adjuvant for 2 months.

Abbreviations used: ACBP, acyl-CoA-binding protein; FABP, fatty acid-binding protein; BSA, bovine serum albumin; PBS, phosphate-buffered saline [20 mM-potassium phosphate (pH 7.4)/150 mM-NaCl]; ELISA, enzyme-linked immunosorbent assay.

Antibody titre against ACBP was determined by ELISA (Tanaka *et al.*, 1985). Micro-titre plates were coated with ACBP (0.4 μg) in 100 μl of 50 mM-Na_2CO_3 buffer (pH 9.5)/well, overnight. After unbound ACBP was washed out with PBS, plates were incubated with a blocking solution (1% BSA in PBS) for 3 h at 37 °C to prevent non-specific binding of antiserum. Antiserum diluted in 0.1% BSA in PBS was added and incubation carried out overnight. Antibody binding was detected by alkaline phosphatase-conjugated affinity-purified pig anti-rabbit IgG. Binding was detected by addition of 200 μl of substrate solution [1.0 mg of *p*-nitrophenyl phosphate in 0.1 M-ethanolamine (pH 9.8)/0.5 μM-$MgCl_2$]. Preimmune serum from the rabbit served as a negative control and gave readings which were less than 1% of those with immune serum at the same dilutions.

Affinity purification of anti-ACBP antibodies

ACBP (4.0 mg) was coupled to 1 g of CNBr-activated Sepharose 4B essentially as recommended by the manufacturer (Pharmacia). The ACBP-specific antibody was purified on this column.

Preparation of subcellular fractions

A 20% (w/v) homogenate of cow liver was prepared in 0.25 M-sucrose/20 mM-potassium phosphate buffer (pH 7.2)/1 mM-EDTA. Subcellular fractions were obtained by differential centrifugation: nuclei and cell debris (10 min at 700 *g*), mitochondria (10 min at 8000 *g*), microsomal fraction (60 min at 105000 *g*) and the membrane-free cytosol. The nuclei and cell debris fraction and the mitochondria fraction were resuspended in the homogenization buffer and centrifuged once more as described above. The tissues in which the concentration of ACBP was to be measured were homogenized in 2 vol. (v/w) of 100 mM-potassium phosphate buffer (pH 7.0)/1.0 mM-EDTA with a Potter–Elvehjem homogenizer and centrifuged for 20 min at 10000 *g*. The supernatant was used for ELISA and immunoblotting without further treatment.

Blotting and immunological detection of proteins

Polyacrylamide-gel electrophoresis was carried out essentially as described by Laemmli (1970), modified as described by Grunnet & Knudsen (1978). The slab gels contained 20% acrylamide. The M_r markers used were prestained protein M_r standards, of M_r range 14300–200000.

After electrophoresis the proteins were electrophoretically transferred from the gels to nitrocellulose sheets exactly as described by Burnette (1981), with a transfer time of 4 h at a current of 0.4 A. After transfer, the blots were immersed in 100 ml of PBS containing 3% (w/v) BSA and incubated at 37 °C for 3 h on a rocking platform. Next, the blot was transferred to a solution of 100 ml of PBS containing 3% BSA, 0.05% (v/v) Tween-20 and 2.8 μg of affinity-purified anti-ACBP IgG/ml, and incubated overnight at room temperature. After washing for 3 × 5 min with PBS containing 0.05% Tween-20, the blot was transferred to 100 ml of PBS containing 3% BSA, 0.05% Tween-20 and alkaline phosphatase-conjugated affinity-purified swine anti-rabbit IgG (1:1000 dilution) and incubated for 3 h at room temperature. After washing for 3 × 5 min with 100 ml of PBS containing 0.05% Tween-20, and once with 100 ml of 0.1 M-ethanolamine, pH 9.0, the alkaline phosphatase activity was detected by the method of Blake *et al.* (1984). The reaction was quantified as described by Blake *et al.* (1984), with a Shimadzu CS-930 t.l.c. scanner, and areas were integrated with a Shimadzu DA-2 data recorder.

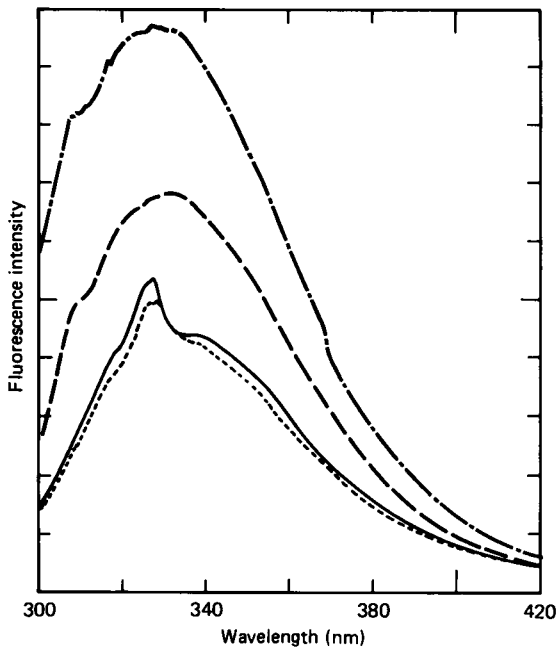

Fig. 1. Fluorescence emission spectra for ACBP excited at 280 nm and 296 nm with and without added decanoyl-CoA

ACBP (0.12 mg/1.2 ml) was solubilized in 10 mM-potassium phosphate buffer, pH 7.0, and decanoyl-CoA (14 nmol) was added in 20 μl of the same buffer. ——— and —·—·—, Emission spectra for excitation at 280 nm with and without 11.7 μM-decanoyl-CoA added respectively. ---- and ——, Emission spectra for excitation at 296 nm with and without 11.7 μM-decanoyl-CoA added respectively.

ELISA of ACBP

ACBP was assayed by a multiple-layer enzyme-linked immunochemical procedure as described by Pawlak & Smith (1986). The layers, attached sequentially to a 96-well plastic plate, consisted successively of monospecific rabbit anti-ACBP IgG, ACBP antigen, biotinylated rabbit anti-ACBP IgG, avidin and biotinylated alkaline phosphatase; the chromogenic substrate was *p*-nitrophenyl phosphate.

Protein determined

The proteins were precipitated with 15% (v/v) trichloroacetic acid and measured by the method of Lowry *et al.* (1951), with BSA as standard.

RESULTS AND DISCUSSION

Binding characteristics

The fluorescence emission spectra for ACBP were only slightly affected by decanoyl-CoA when 296 nm was used as the excitation wavelength (Fig. 1). The intrinsic fluorescence at 332 nm with excitation at 296 nm is

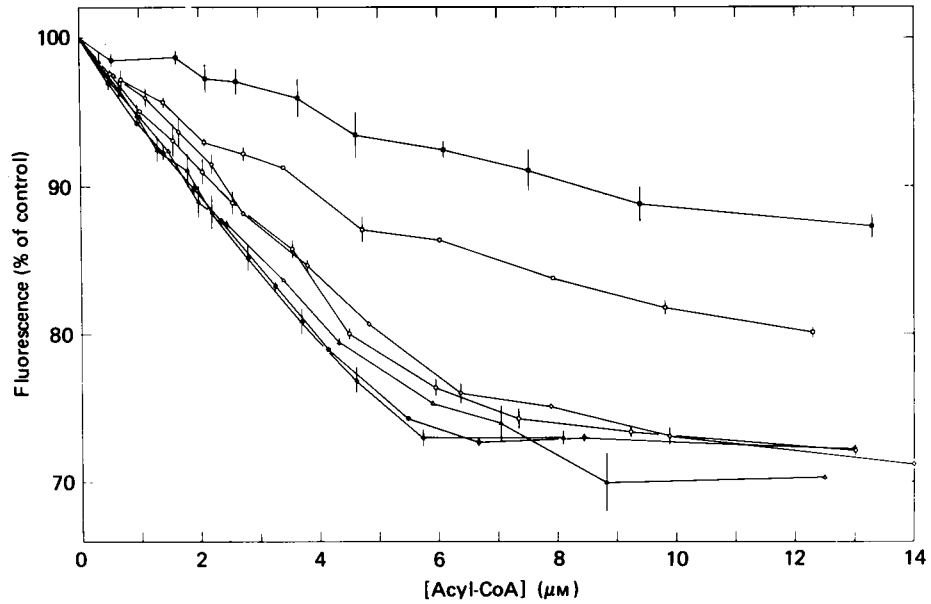

Fig. 2. Acyl-CoA quenching of the intrinsic fluorescence of ACBP

ACBP in 10 mM-potassium phosphate buffer, pH 7.0, was excited at 280 nm, and fluorescence was measured at 332 nm in the absence and presence of different concentrations of acyl-CoA: ■, acetyl-CoA; □, butyryl-CoA; ◇, octanoyl-CoA; △, decanoyl-CoA; ●, dodecanoyl-CoA; ▲, tetradecanoyl-CoA; ○, hexadecanoyl-CoA. The results show fluorescence expressed as a percentage of that in controls without added acyl-CoA esters. For further details see the Materials and methods section. The results are means of the duplicate determinations with ± half the difference shown by bars.

Table 1. Relative affinity of ACBP for different chain-length acyl-CoA esters, calculated as the slope of plots of percentage inhibition of fluorescence against acyl-CoA concentration

The data used for calculation are taken from Fig. 2.

Acyl-CoA	Slope (% inhibition of fluorescence · μM^{-1})
Acetyl-CoA	1.23
Butyryl-CoA	2.39
Octyl-CoA	4.00
Decanoyl-CoA	4.56
Dodecanoyl-CoA	4.80
Tetradecanoyl-CoA	5.03
Hexadecanoyl-CoA	4.12

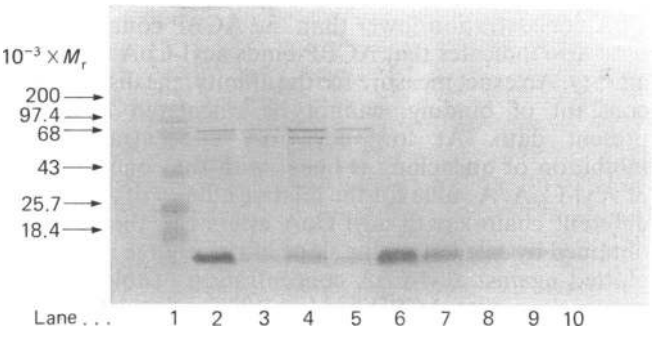

Fig. 3. Immunostaining with anti-ACBP antibodies of a Western blot of different liver subcellular fractions and ACBP

Liver subcellular fractions and purified ACBP were separated on a 20%-polyacrylamide gel. The gel was then electrophoretically blotted on to nitrocellulose, and the resulting blot was processed as described in the Materials and methods section. Lanes contained the following samples: 1, prestained standard proteins; 2, 105000 *g* supernatant (24 μg of protein); 3, mitochondria (23 μg of protein); 4, microsomal fraction (32 μg of protein); 5, nuclei and cell debris (24 μg of protein); 6, ACBP (100 ng); 7, ACBP (50 ng); 8, ACBP (25 ng); 9, ACBP (13 ng); 10, ACBP (6.3 ng).

almost exclusively due to tryptophan (Lakowicz, 1983). In contrast, the intrinsic emission at 332 nm was decreased by up to 30% upon decanoyl-CoA addition, when 280 nm was used as the excitation wavelength. The above result strongly indicates that decanoyl-CoA interacts with tyrosine rather than tryptophan upon binding. An alternative explanation could be that decanoyl-CoA changes the protein conformation upon binding and thereby changes tyrosine fluorescence.

The ability of individual acyl-CoA esters to quench the intrinsic fluorescence at 332 nm from ACBP excited at 280 nm was used as a measurement for the binding affinity of the protein for acyl-CoA esters of different chain length (Fig. 2). All the tested acyl-CoA esters with an acyl chain length $> C_8$ effectively quenched fluorescence. The results in Fig. 2 also show that maximum quenching by dodecanoyl-CoA and tetradecanoyl-CoA is obtained with a concentration of 5–6 μM. This is about half the concentration of ACBP in the incubation mixture. If it is assumed that ACBP is saturated with ligand when the maximum quenching is obtained, the binding stoichiometry must be 1 molecule of acyl-CoA/2 molecules of ACBP. This indicates that ACBP appears

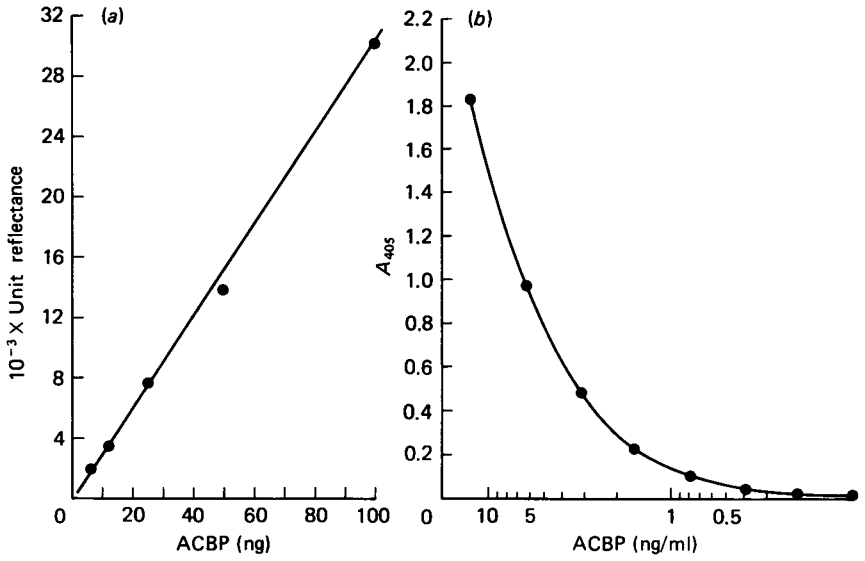

Fig. 4. Standard curve for ACBP assays by immunoblotting (*a*) and ELISA (*b*)

Each point represents a single determination. See the Materials and methods section for further details.

as a dimer in solution or that ligand binding induces dimer formation. Self-aggregation has also been shown to occur in solution of cardiac FABP (Fournier *et al.*, 1983; Fournier & Rahin, 1983).

The fact that maximum binding is obtained at an acyl-CoA concentration lower than the ACBP concentration used also indicates that ACBP binds acyl-CoA with high affinity. An exact measure for the affinity, the dissociation constant of binding, cannot be calculated from the present data. At low acyl-CoA concentration the inhibition of quenching is linear with the concentration of acyl-CoA. A value for the relative affinity of ACBP for different chain-length acyl-CoA esters can therefore be obtained by calculating the slope of percentage inhibition plotted against acyl-CoA concentration (Table 1). The results show that ACBP has low affinity for acetyl-CoA and butyryl-CoA and about equal affinity for $C_{12:0}$ to $C_{16:0}$ acyl-CoA esters.

As the acyl-CoA binding to ACBP is dependent on the chain length of acyl-CoA, the binding must involve a hydrophobic interaction. Therefore the binding of non-esterified fatty acids was examined by incubation of ACBP with increasing amount of decanoic acid. Decanoic acid was unable to quench the intrinsic fluorescence of ACBP. Furthermore, the presence of decanoic acid did not affect quenching by decanoyl-CoA (results not shown). Another approach was to use dansyl-undecanoic acid as a probe for fatty acid binding to ACBP. The binding of this compound to rat liver FABP is accompanied by a shift in its fluorescence emission maximum from 550 to 500 nm and a 60-fold enhancement of its fluorescence intensity at 500 nm (Wilkinson & Wilton, 1986). It was not possible to detect any change in fluorescence intensity of dansyl-undecanoic acid, when incubated with ACBP (results not shown). This indicated that ACBP cannot bind non-esterified fatty acids, and this conclusion is in agreement with the data of Mogensen *et al.* (1987), where the fraction which contained the ACBP did not show any fatty acid-binding activity. This makes ACBP different from FABP, which binds both fatty acids and acyl-CoA esters (Glatz & Veerkamp, 1984).

Intracellular and tissue distribution of ACBP

In order to gain a better understanding of the function of ACBP, we determined its intracellular localization and its distribution among different tissues in the cow. The determination of ACBP in subcellular fractions obtained by differential centrifugation was done by quantitative Western blots. A typical blot of subcellular fractions is shown in Fig. 3, and the linearity and sensitivity of the method are shown in Fig. 4(*a*). Fig. 3 shows that the affinity-purified anti-ACBP IgG slightly cross-reacts with a number of other proteins, especially in the microsomal fraction. Some of these proteins also cross-reacted with preimmune serum, but no protein close to the M_r of ACBP was stained, when this serum

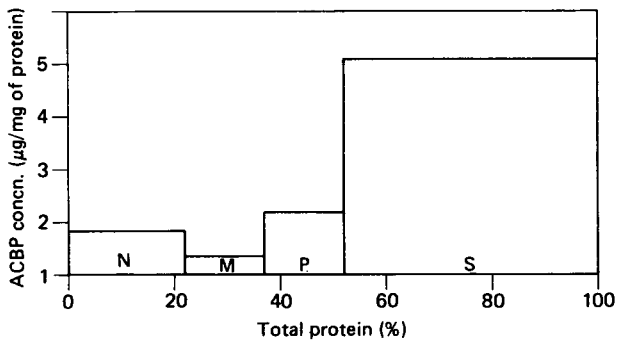

Fig. 5. Subcellular distribution of ACBP in cow liver

A homogenate of a non-perfused liver in 0.25 M-sucrose was fractionated into a nuclei and cell debris pellet (N), mitochondrial pellet (M), microsomal pellet (P) and a 105000 *g* supernatant (S). The resulting fractions were assayed for ACBP by Western blotting. The results are means of two determinations. For further details see the Materials and methods section.

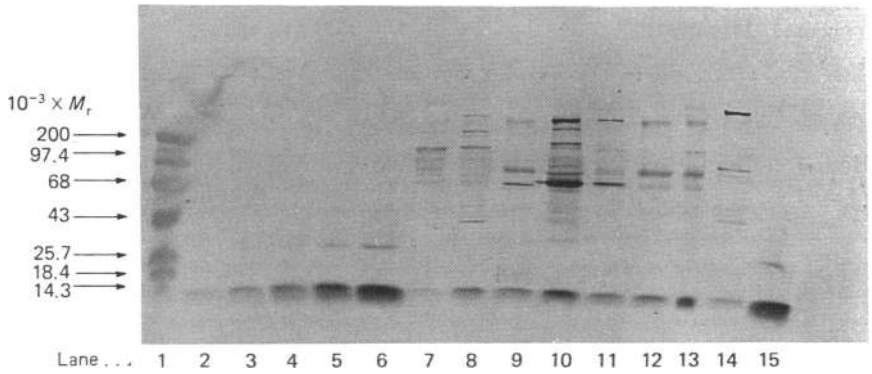

Fig. 6. Tissue distribution of antigen recognized by anti-ACBP antibodies in Western blotting analysis

Extracts from various cow tissues prepared as described in the Materials and methods section were separated on a 20%-polyacrylamide gel. After transfer to the nitrocellulose membrane, the antigen was detected by immunostaining. Lanes contained the following samples: 1, prestained standard proteins; 2, ACBP (6.3 ng); 3, ACBP (13 ng); 4, ACBP (25 ng); 5, ACBP (50 ng); 6, ACBP (100 ng); 7, mammary gland (15 μg of protein); 8, brain (18 μg of protein); 9, intestinal mucosa (16 μg of protein); 10, liver (16 μg of protein); 11, heart (15 μg of protein); 12, kidney, (16 μg of protein); 13, adipose tissue (15 μg of protein); 14, muscle (15 μg of protein); 15, ACBP (100 μg). For experimental details see the Materials and methods section.

Table 2. ACBP contents in various cow tissues

Values represent means of duplicate determinations ± half differences. For experimental details see the Materials and methods section.

Tissue	ACBP (μg/mg of protein) measured by: Immunoblot	ELISA
Liver	3.3±0.5	2.5±0.1
Adipose	1.8±0.1	2.0±0.2
Intestinal mucosa	1.5±0.1	1.1±0.1
Kidney	1.4±0.1	1.2±0.1
Heart	1.2±0.0	0.71±0.04
Brain	1.2±0.3	1.2±0.3
Muscles	0.61±0.07	0.84±0.12
Mammary gland	0.24±0.04	0.64±0.07

was used. About 82% of the ACBP present in cells was found in the 105000 *g* supernatant (Fig. 5). The ACBP contents in different cow tissues were measured both by quantitative Western blotting and by sandwich ELISA. The sensitivity of the sandwich ELISA used is shown in Fig. 4(*b*), and a typical immunostained Western blot of the 10000 *g* supernatant from different cow tissues is shown in Fig. 6. The second band appearing on the blots of purified ACBP is caused by dimer formation upon storage. This blot also shows the cross-reactivity of the affinity-purified anti-ACBP IgG with other proteins. The relative amount of ACBP found in different tissues was similar in both methods (Table 2). In most tissues the amount determined by the ELISA method was slightly lower than that determined by the Western-blotting method. The fact that the content in liver is lower with the ELISA method indicates that the cross-reactivity of the IgG used is unimportant. The highest concentration of ACBP was found in liver, but significant amounts were found in all tissues tested, including brain and mammary glands.

The above results show that ACBP is a cytosolic protein similar to FABP (Rustow *et al.*, 1979). The concentration (3 μg/mg of protein) of ACBP in cow liver is about 10 times lower than that of FABP in rat liver (Ockner *et al.*, 1982).

It is interesting to speculate that ACBP acts as an intracellular carrier of acyl-CoA, as has been suggested for FABPs with regard to intracellular transport of fatty acids (Glatz & Veerkamp, 1984). The ACBP might also act as a sink, preventing cellular acyl-CoA being dissolved in cellular membranes and thereby causing a disturbing detergent effect. In this connection it is interesting that the concentration of long-chain acyl-CoA in rat liver (108–248 nmol/g of protein; Woldegiorgis *et al.*, 1985) is in the same range as the concentration of ACBP, 250–330 nmol/g of protein. To what extent the ACBP–acyl-CoA complex can act as a substrate for different lipogenic enzymes and to what extent ACBP can compete with cellular membranes for acyl-CoA will have to await further experimental testing.

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