Interaction of acyl-CoA binding protein (ACBP) on processes for which acyl-CoA is a substrate, product or inhibitor

Jan Trige RASMUSSEN, Jesper ROSENDAL and Jens KNUDSEN*
Institute of Biochemistry, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

It is shown that acyl-CoA binding protein (ACBP), in contrast with fatty acid binding protein (FABP), stimulates the synthesis of long-chain acyl-CoA esters by mitochondria. ACBP effectively opposes the product feedback inhibition of the long-chain acyl-CoA synthetase by sequestration of the synthesized acyl-CoA esters. Feedback inhibition of microsomal long-chain acyl-CoA synthesis could not be observed, due to the formation of small acyl-CoA binding vesicles during preparation and/or incubation. Microsomal membrane preparations are therefore unsuitable for studying feedback inhibition of long-chain acyl-CoA synthesis. ACBP was found to have a strong attenuating effect on the long-chain acyl-CoA inhibition of both acetyl-CoA carboxylase and mitochondrial adenine nucleotide translocase. Both processes were unaffected by the presence of long-chain acyl-CoA esters when the ratio of long-chain acyl-CoA to ACBP was below 1, independent of the acyl-CoA concentration used. It is therefore not the acyl-CoA concentration as such which is important from a regulatory point of view, but the ratio of acyl-CoA to ACBP. The cytosolic ratio of long-chain acyl-CoA to ACBP was shown to be well below 1 in the liver of fed rats. ACBP could compete with the triacylglycerol-synthesizing pathway, but not with the phospholipid-synthesizing enzymes, for acyl-CoA esters. Furthermore, in contrast with FABP, ACBP was able to protect long-chain acyl-CoA esters against hydrolysis by microsomal acyl-CoA hydrolases. The results suggest that long-chain acyl-CoA esters synthesized for either triacylglycerol synthesis or β-oxidation have to pass through the acyl-CoA/ACBP pool before utilization. This means that acyl-CoA synthesized by microsomal or mitochondrial synthetases is uniformly available in the cell. It is suggested that ACBP has a duel function in (1) creating a cytosolic pool of acyl-CoA protected against acyl-CoA hydrolases, and (2) protecting vital cellular processes from being affected by long-chain acyl-CoA esters.

INTRODUCTION

Acyl-CoA binding protein (ACBP) is a highly conserved cytosolic protein, consisting of 86 amino acid residues with an acylated N-terminus (Mikkelsen et al., 1987; Knudsen et al., 1989). ACBP was found to bind acyl-CoA esters with chain length from C_{14} to C_{22} with high affinity (Mikkelsen and Knudsen, 1987; Rosendal et al., 1993). By using photoreactive acyl-CoA esters, the hydrophobic binding site on bovine ACBP was determined to include amino acids 23–38 (Hach et al., 1990), which was assigned to the second of four alpha-helices (Andersen et al., 1991).

ACBP has also been suggested to be a modulator of neuroactivity, to be involved in regulation of steroid hormone synthesis and regulation of insulin secretion (for a review see Knudsen et al., 1993). However, conclusive evidence for the involvement of ACBP in these functions is still to be found (Knudsen and Nielsen, 1990).

That ACBP participates in acyl-CoA metabolism is supported by its ability to bind with high affinity and to sequester long-chain acyl-CoA esters (Mikkelsen and Knudsen, 1987; Rasmussen et al., 1990; Rosendal et al., 1993). These observations, and the fact that the genomic gene of ACBP has all the characteristics of a housekeeping gene (Mandrup et al., 1992), indicate that ACBP is a housekeeping protein involved in acyl-CoA metabolism and not a protein with specific functions expressed in specific tissues. The observation that the pool of long-chain acyl-CoA esters is dramatically increased when ACBP is over-expressed in yeast shows that ACBP also binds acyl-CoA esters *in vivo* and thereby forms a pool of long-chain acyl-CoA esters (Mandrup et al., 1993).

Activation of long-chain fatty acids catalysed by the long-chain acyl-CoA synthetase (lc-ACS, EC 6.2.1.3) is the first reaction in fatty acid metabolism. Mitochondria, endoplasmic reticulum and peroxisomes all contain ATP-dependent lc-ACS activity (Shindo and Hashimoto, 1978; Krisans et al., 1980; Singh et al., 1985). This enzyme is membrane-bound in all three organelles, and the active sites of the enzyme face the cytosol in the endoplasmic reticulum and peroxisomes (Singh et al., 1985; Mannaerts et al., 1982). The mitochondrial synthetase is an integral part of the outer membrane, exposing its CoA-binding site to the cytosol (Hesler et al., 1990). The properties of the enzymes in the rat liver mitochondria, microsomes and peroxisomes were indistinguishable, indicating that it is one and the same enzyme (Tanaka et al., 1979; Miyazawa et al., 1985). This is further supported by the observation that lc-ACS in the three organelles has the same amino acid composition (Suzuki et al., 1990).

In spite of the central role of the enzyme in fatty acid metabolism, little is known about its regulation. However, long-chain acyl-CoA synthesis was shown to be feedback-regulated by hexadecanoyl-CoA, with an apparent K_i of 4 μM (Pande, 1973).

A large number of cellular processes have been reported to be affected by long-chain acyl-CoA esters (see, e.g., Wise and Kuo, 1983; Powell et al., 1985; Pfanner et al., 1989; Prentki et al., 1992). Two proteins in particular have been shown to be inhibited by extremely low concentrations of long-chain acyl-CoA esters, namely acetyl-CoA carboxylase (ACC; K_i = 1–5 nM; Nikawa et al., 1979) and adenine nucleotide translocase (ANT; $K_i < 1$ μM; Woldegiorgis et al., 1982). Consequently long-chain acyl-CoA esters would, if not sequestered in some way, be expected to

Abbreviations used: ACBP, acyl-CoA binding protein; ACC, acetyl-CoA carboxylase; ANT, adenine nucleotide translocase; L-FABP, liver fatty acid binding protein; lc-ACS, long-chain acyl-CoA synthetase.

* To whom correspondence should be addressed.

suppress fatty acid synthesis *de novo* and/or to inhibit ATP synthesis by affecting the transport of adenine nucleotides across the mitochondrial inner membrane.

In the present paper, we have investigated the effects of ACBP and liver fatty acid binding protein (L-FABP) on long-chain acyl-CoA synthesis by mitochondria and microsomal membranes. The results show that ACBP stimulates mitochondrial acyl-CoA synthetase activity in the presence of FABP. We have investigated the role of long-chain acyl-CoA esters as regulators of ACC and ANT and present data on the long-chain acyl-CoA ester/ACBP ratio in fed-rat liver. The results suggest that ACC and ANT are not regulated by long-chain acyl-CoA ester in fed-rat liver.

MATERIALS AND METHODS

Materials

Sprague–Dawley male rats (200 g) and lactating rabbits (2–3 weeks *post partum*) were used. [1-^{14}C]Hexadecanoic acid was from Amersham International. ATP, BSA and L-glycerol 3-phosphate were supplied by Boehringer, Mannheim, Germany. High-performance-t.l.c. silica-gel 60 plates were from E. Merck, Darmstadt, Germany. CoA and Percoll were from Pharmacia, Uppsala, Sweden. Acetonitrile was purchased from Rathburn Chemicals, Walkerburn, Scotland, U.K. Nucleosil ODS (10 μm) packing was from Machery–Nagel, Düren, Germany. All other chemicals used were of analytical grade, supplied by either Merck or Sigma Chemical Co., St. Louis, MO, U.S.A.

Purification of ACBP and FABP

Recombinant ACBP was obtained by the procedure of Mandrup et al. (1991). L-FABP was prepared from bovine liver as described by Rasmussen et al. (1990).

Preparation of rat liver mitochondria and microsomal membranes

Rat liver was homogenized in 50 ml of 225 mM mannitol/75 mM sucrose/1 mM EGTA/5 mM potassium phosphate buffer, pH 7.4, and mitochondria were obtained by the procedure of Reinhart et al. (1982), using discontinuous isopycnic Percoll-gradient centrifugation. Respiratory control index was at least 6 for all mitochondrial preparations used. Rat liver microsomal membranes were obtained as described by Rasmussen et al. (1990) and the rabbit mammary-gland microsomal membranes were prepared as described by Knudsen (1976).

Protein determination

The concentration of recombinant ACBP was determined by using $\epsilon_{280} = 15.4\ mM^{-1} \cdot cm^{-1}$, as determined for native bovine ACBP (Rasmussen et al., 1990). The amount of protein in mitochondrial and microsomal membranes was determined by the method described by Schachterle and Pollack (1973), with BSA as standard.

Synthesis of acyl-CoA

Hexadecanoyl-CoA, *cis*-9-[1-^{14}C]octadecenoyl-CoA and *S*-hexadecyl-CoA were prepared by the procedures of Rosendal et al. (1993). Acetyl-CoA was prepared by the method of Smith et al. (1966) and analysed for purity by using the h.p.l.c. conditions described under 'Activity of ACC'.

Assay of long-chain acyl-CoA synthesis

The reaction mixture contained final concentrations of 350 mM Tris/HCl (pH 7.4), 8 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM CoA, 10 mM ATP, 0.8% (v/v) dimethyl sulphoxide, 20 μM [1-^{14}C]hexadecanoic acid (sp. radioactivity 56 Ci/mol), and the indicated amount of ACBP and/or L-FABP. Dimethyl sulphoxide does not affect lc-ACS when the final concentration is kept below 1% (Morand and Aigrot, 1985).

After 5 min of preincubation at 37 °C, the reaction was initiated by addition of the freshly prepared mitochondrial or microsomal fraction, giving a final concentration of 5 μg of mitochondrial/microsomal protein/ml. The assay mixtures were incubated for the indicated time at 37 °C, and stopped by addition of 6 vol. of chloroform/methanol (1:1, v/v). One portion of each sample was analysed for radioactivity by scintillation counting, and a second sample by high-performance t.l.c. The samples were applied on the plates by using a Camag Linomat IV instrument. The silica-gel plates were developed with chloroform/methanol/acetic acid/water (50:25:7:3, by vol.) and scanned for radioactivity by using a Berthold Automatic linear t.l.c. analyser. The solvent system used separates acyl-CoA esters, phospholipids and neutral lipids totally. The [1-^{14}C]hexadecanoyl-CoA band was identified by running an authentic standard. The radioactivity incorporated into acyl-CoA was calculated from the distribution of radioactivity on the plates, knowing the total amount of radioactivity loaded on the plates.

To estimate the distribution of fatty acids and acyl-CoA between the incubation medium and the mitochondria, samples were incubated for 15 min at 37 °C under the conditions described above, and stopped immediately by cooling them to 0 °C. The samples were centrifuged at 22000 ***g*** for 15 min at 0 °C. A sample of the resulting supernatant and of the remaining part of the supernatant plus pellet was analysed for radioactivity as described above after addition of chloroform/methanol. The distribution of substrate and product could then be determined.

Purification of ACC

ACC was prepared from the mammary gland of lactating rabbits by the method of Tipper and Witters (1982) or obtained as a gift from Dr. D. G. Hardie, University of Dundee, U.K. The specific activity of the ACC used was between 0.8 and 1.2 μmol of malonyl-CoA synthesized/min per mg.

Activity of ACC

ACC activity was assayed in 100 mM potassium phosphate (pH 7.4)/25 mM potassium citrate/20 mM $MgCl_2$/2 mM ATP/0.35 mM acetyl-CoA/20 mM $NaHCO_3$, plus 70 nM ACC. The samples were incubated with the indicated amount of hexadecanoyl-CoA and ACBP. After preincubation for 15 min at 37 °C the reaction was initiated by adding ATP and acetyl-CoA. The samples were stopped after 90 s by transferring them to a hot water bath (95 °C) for 100 s. The samples were then centrifuged at 20000 ***g*** for 1 min and stored at −80 °C until analysis. The activity was determined by measuring the amount of malonyl-CoA synthesized by reverse-phase h.p.l.c. on a column (250 mm × 4 mm) packed with Nucleosil ODS. The column was equilibrated in 95% buffer A (25 mM ammonium acetate,

pH 5.3) and 5% buffer B [15% (v/v) acetonitrile in buffer A] with a flow rate of 1.0 ml/min. The short-chain acyl-CoA esters were eluted with a linear gradient of 5–50% buffer B in 30 min, 5 min 50% buffer B, and back to 5% buffer B in 5 min. Amounts of malonyl-CoA synthesized were calculated from a standard curve relating the injected amount of malonyl-CoA to the resulting peak area.

Activity of ANT

Oxygen-electrode measurements were performed as described by Lessler and Brierley (1969). Respiration was initiated by adding succinate (10 mM) and the State-3 respiration was accomplished by adding ADP (2 mM). The concentration of mitochondrial protein was 1 mg/ml. Other conditions used were in accordance with Estabrook (1967).

Synthesis of glycerolipids in rabbit mammary-gland microsomes

The incubation medium consisted of 10 mM Tris/HCl (pH 7.4), 100 mM NaCl, 2 mM ATP, 0.5 mM CoA, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM [1-^{14}C]hexadecanoic acid (sp. radioactivity 56 Ci/mol), 10 μM L-FABP, 2 mM glycerol 3-phosphate, 70 μg/ml microsomal protein, and the indicated amounts of ACBP. After 5 min of preincubation at 37 °C the reaction was started by addition of fatty acids and L-FABP. The reaction was stopped with 6 vol. of chloroform/methanol (1:1, v/v) after 15 min of incubation at 37 °C. The glycerolipid classes were separated by high-performance silica-gel t.l.c. plates, using two developing solvents. First acyl-CoA esters, phospholipids and neutral lipids were separated by running the plates half-way in chloroform/methanol/acetic acid/water (50:25:7:3, by vol.). The plates were then dried and the neutral lipids were further separated with hexane/diethyl ether/methanol/acetic acid (90:20:3:2, by vol.). The plates were scanned on a Berthold Automatic t.l.c. linear analyser. The lipids and long-chain acyl-CoA esters were identified by running authentic standards. Radioactivity incorporated was quantified from the distribution of radioactivity on the t.l.c. plates, knowing the total amount of radioactivity loaded as determined by liquid-scintillation counting.

Microsomal hydrolysis of long-chain acyl-CoA esters

Rat liver microsomal membranes (300 μg of protein/ml) were incubated with *cis*-9-[1-^{14}C]octadecenoyl-CoA (5 μM; sp. radioactivity 10 Ci/mol) for 70 min at 5 °C in 10 mM Tris/HCl (pH 7.4)/100 mM NaCl with or without 5 μM of L-FABP or ACBP. The amount of acyl-CoA hydrolysis was determined by high-performance t.l.c. as described above after addition of 6 vol. of chloroform/methanol (1:1, v/v).

Extraction and measurements of long-chain acyl-CoA esters

Long-chain acyl-CoA esters from rat liver were extracted and quantified as described by Rosendal and Knudsen (1992). Mitochondria were isolated and purified as described above, and the recovery was calculated from the recovery of cytochrome *c* oxidase (Hesler et al., 1990). The mitochondrial suspension (4.0 ml; 50.37 mg/ml) was diluted with water (3.5 ml) and the long-chain acyl-CoA esters were quantified as described above. To prevent metabolism of the acyl-CoA esters, the temperature was kept low (0–4 °C) during mitochondrial preparation (20 min).

Determination of ACBP levels by e.l.i.s.a.

ACBP was measured by the multiple-layer linked immunochemical procedure described by Knudsen et al. (1989).

RESULTS AND DISCUSSION

Effects of ACBP and FABP on mitochondrial lc-ACS activity

Mitochondria prepared by using Percoll-density-gradient centrifugation were used as source of lc-ACS. The synthesis of hexadecanoyl-CoA was linear for 15 min with 5 μg/ml mitochondrial protein.

ACBP (30 μM) significantly stimulated lc-ACS activity (Figure 1). The initial rate was more than doubled in the presence of ACBP, to 102 nmol/min per mg, compared with 46 nmol/min per mg in the control. FABP has been claimed to stimulate lc-ACS (Wu-Rideout et al., 1976; Burnett et al., 1979; Burrier et al., 1987). We therefore tested the effect of bovine L-FABP on mitochondrial lc-ACS as well. L-FABP (30 μM) did not influence the activity of the synthetase (Figure 1). The initial rate was nearly the same in the presence of L-FABP (45 nmol/min per mg) as in the control. The lc-ACS activity was also studied in presence of both ACBP and L-FABP (Figure 1). With both proteins present, the activity (94 nmol/min per mg) was similar to that obtained with ACBP alone.

The localization of substrate and product was analysed to obtain evidence as to whether the effect of ACBP on lc-ACS activity was due to removal of inhibitory long-chain acyl-CoA from the mitochondria. At the end of the incubation, the samples were immediately cooled to 0 °C. The mitochondria were spun

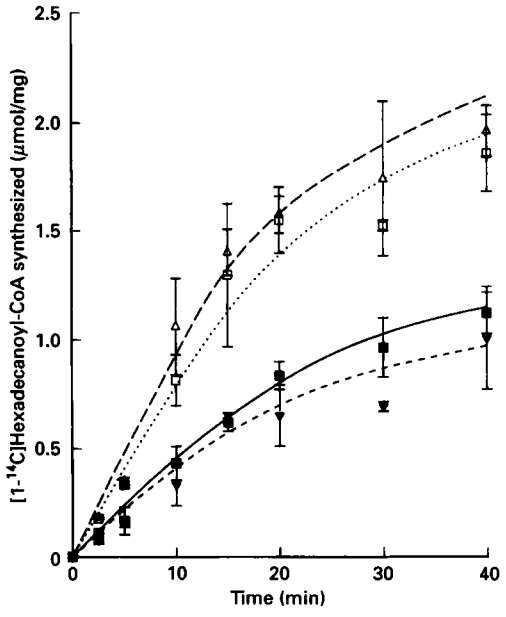

Figure 1 Long-chain acyl-CoA synthesis by mitochondria

Assays for lc-ACS activity employing [1-^{14}C]hexadecanoic acid (20 μM) and mitochondria (5 μg of protein/ml) were performed as described in the Materials and methods section. The results are representative of individual experiments and are plotted as mean ± range of duplicates. Effects of binding proteins (30 μM of each) on lc-ACS activity were measured: ■, no binding proteins; △, ACBP; ▼, L-FABP; □, ACBP + L-FABP.

Table 1 Localization of synthesized [1-^{14}C]hexadecanoyl-CoA

Long-chain acyl-CoA synthesis was measured in the absence (control) or in the presence of ACBP and/or FABP (30 μM of each). At the end of the incubation (15 min) the samples were cooled to 0 °C. The mitochondria were spun down, and the distribution of hexadecanoic acid and hexadecanoyl-CoA between the incubation medium (free) and the mitochondria (bound) was determined as described in the Materials and methods section. The results are representative for individual experiments and are given as mean ± range of triplicates.

Condition		Hexadecanoyl-CoA (pmol)	Hexadecanoic acid (pmol)
Control	Free	91.0 ± 23.5	220.7 ± 22.6
	Bound	89.3 ± 12.3	599.0 ± 2.2
ACBP	Free	342.2 ± 30.0	136.0 ± 0.1
	Bound	0.3 ± 0.2	524.0 ± 27.2
FABP	Free	156.1 ± 18.6	806.8 ± 3.2
	Bound	32.4 ± 11.1	6.0 ± 4.6
ACBP + FABP	Free	328.0 ± 31.0	657.9 ± 23.3
	Bound	1.9 ± 1.4	5.0 ± 4.9

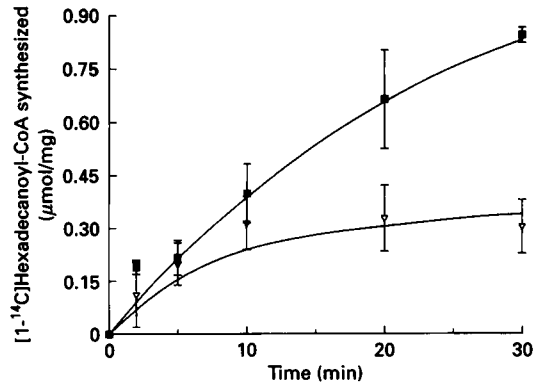

Figure 2 Activity of lc-ACS in presence of hexadecanoyl-CoA/ACBP complex (1:1, mol/mol)

The activity of lc-ACS was measured in the absence (■) or in the presence (▽) of hexadecanoyl-CoA/ACBP complex (30 μM). The results are representative of individual experiments and are shown as mean ± range of duplicates. For further details see the Materials and methods section.

down, and the amounts of hexadecanoyl-CoA and hexadecanoic acid in the supernatant and the pellet were determined (Table 1). The results clearly demonstrate that ACBP solubilizes all the hexadecanoyl-CoA produced by the mitochondrial lc-ACS. without affecting the fatty acid distribution significantly. This strongly indicates that the stimulation of lc-ACS by ACBP was due to removal of inhibitory long-chain acyl-CoA from the mitochondria, as lc-ACS has been shown to be product-inhibited (Pande, 1973). L-FABP solubilizes all the fatty acids and a part of the hexadecanoyl-CoA synthesized, but it had no effect on the rate, indicating that L-FABP can desorb loosely bound acyl-CoA, but not acyl-CoA placed on the leaving site of lc-ACS. The fact that L-FABP solubilizes all the substrate without affecting the rate strongly indicates that L-FABP can deliver fatty acids directly to lc-ACS. In the presence of both L-FABP and ACBP, practically all of the substrate and product was found in the incubation medium.

The present results clearly, for the first time, demonstrate co-operativity between L-FABP and ACBP function. L-FABPs facilitate the diffusion of fatty acids through the aqueous medium to the membrane-bound enzymes, thus helping to maintain the concentration of this poorly water-soluble compound at an optimum close to the active sites of the enzymes. ACBP, by its ability to sequester long-chain acyl-CoA esters, contributes by releasing product inhibition of ACS. Together the two proteins have a similar effect, as observed with albumin alone in several enzyme assays (Burnett et al., 1979; Jamdar, 1979; Richards et al., 1991).

If ACBP stimulates acyl-CoA synthesis by removing long-chain acyl-CoA esters from lc-ACS, it would be predicted that saturation of ACBP with acyl-CoA would abolish the stimulating effect of ACBP on lc-ACS. When lc-ACS activity was measured in the presence of hexadecanoyl-CoA/ACBP complex (30 μM of each), it was clearly shown that this prediction was correct (Figure 2).

An interesting consequence of the results presented above is that acyl-CoA esters synthesized by mitochondrial lc-ACS will be solubilized in the presence of ACBP. This implies that mitochondrial lc-ACS on the cytosolic face of the outer membrane can contribute to the general pool of long-chain acyl-CoA esters, and does not only activate fatty acids destined for mitochondrial β-oxidation.

The degree of stimulation by ACBP was much smaller when microsomes were used (results not shown), probably because of the artefactual formation of small vesicles, which will act as acceptors for the acyl-CoA esters formed and thereby abolish product inhibition. This notion is supported by the fact that the presence of detergent (1 % tyloxapol) in the incubation completely annuls the effect of ACBP on mitochondrial lc-ACS activity (results not shown). Microsomal fractions are therefore unsuitable for studying product inhibition and the effect of cytosolic proteins on lc-ACS activity.

Effects of ACBP on glycerolipid synthesis

Knowing that newly synthesized acyl-CoA is bound to ACBP even in the presence of small vesicles, we decided to investigate how ACBP would affect microsomal incorporation of newly synthesized acyl-CoA esters into different lipid classes.

The incorporation of hexadecanoyl-CoA into triacylglycerols decreased with increasing amounts of ACBP in the incubation. On the contrary, ACBP did not affect the synthesis of phospholipids significantly (Figure 3). This suggests that phospholipid synthesis is spared at the expense of triacylglycerol synthesis when the acyl-CoA availability is low. The phospholipid synthesis may be directly linked to the synthesis of long-chain acyl-CoA without involving any kind of transport protein, whereas the activity of the triacylglycerol pathway can be influenced by the ratio of long-chain acyl-CoA to ACBP. Therefore it is likely that acyl-CoA synthesized by microsomal lc-ACS for triacylglycerol synthesis is sequestered by ACBP and delivered back to the triacylglycerol-synthesizing enzymes. Thus acyl-CoA esters synthesized by both mitochondrial and microsomal lc-ACS can equally well be used for either microsomal triacylglycerol synthesis or β-oxidation.

Effect of ACBP on hydrolysis of long-chain acyl-CoA esters

Both the cytoplasm and microsomal membranes contain high levels of acyl-CoA hydrolases (Berge, 1979; Berge and Farstad, 1979; Berge et al., 1984). In addition to ACBP's ability to form an intracellular acyl-CoA pool (Mandrup et al., 1993), and its

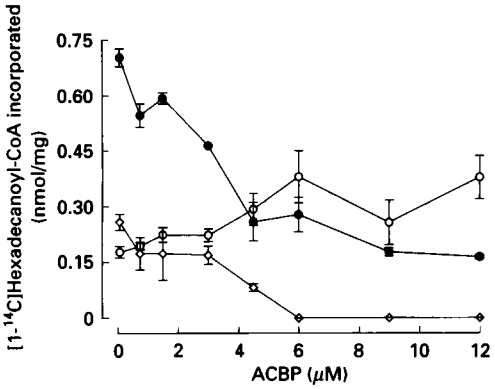

Figure 3 Effects of ACBP on glycerolipid synthesis

Microsomal membranes (70 μg of protein/ml) were incubated for 15 min at 37 °C with increasing amounts of ACBP, and the incorporation of [1-^{14}C]hexadecanoic acid (5 μM initially) into different glycerolipid classes (○, phosphatidylcholine; ◇, triacylglycerol; ●, phosphatidic acid) was determined as described in the Materials and methods section. The results are representative of individual experiments and are depicted as mean ± range of duplicates.

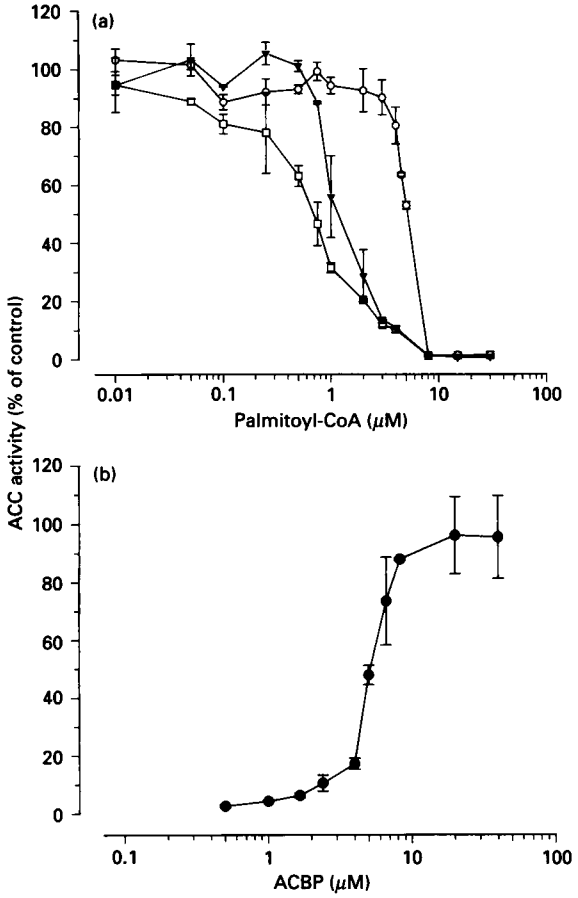

Figure 4 (a) Effects of ACBP on hexadecanoyl-CoA inhibition of ACC and (b) ability of ACBP to reverse hexadecanoyl-CoA-inhibited ACC

(**a**) The ACC activity was measured in assays containing the indicated amounts of hexadecanoyl-CoA. ACC (70 nM) was incubated as described in the Materials and methods section. Results are representative of individual experiments and are plotted as mean of duplicates ± half the difference expressed as percentage of control without hexadecanoyl-CoA added. For further details see the Materials and methods section. □, No ACBP; ▼, 1 μM ACBP; ○, 4 μM ACBP. (**b**) ACC (70 nM) preincubated with hexadecanoyl-CoA (5 μM) was incubated with the indicated amounts of ACBP. Results are representative for individual experiments and are shown as mean of duplicates ± half the difference, expressed as percentage of control with no hexadecanoyl-CoA added. For experimental details, see the Materials and methods section.

ability to affect acyl-CoA synthesis, it might also be able to protect intracellular acyl-CoA esters against hydrolysis. To investigate this phenomenon, we incubated *cis*-9-[1-^{14}C]octadecenoyl-CoA with microsomal membranes and measured acyl-CoA hydrolysis with and without ACBP present. ACBP, in contrast with L-FABP, was able to protect long-chain acyl-CoA from hydrolysis by microsomal membranes. Without ACBP and FABP present, only 10.51 ± 3.05 % of the added acyl-CoA could be recovered, after incubation with microsomal membranes at 0 °C for 70 min. Addition of equimolar amounts of acyl-CoA and ACBP or FABP increased the recovery to 86.07 ± 7.87 % or 17.01 ± 1.86 % respectively. The above results strongly indicate that ACBP is able to create a protected sequestered pool of acyl-CoA. Due to the high binding affinity of ACBP for acyl-CoA ester and the high acyl-CoA hydrolase activity in both membranes and cytosol, the free acyl-CoA concentration is likely to be very small, and this would be expected to affect the ability of long-chain acyl-CoA esters as physiological regulators *in vivo*. We therefore investigated the regulatory role of acyl-CoA in the presence and absence of ACBP.

Effects of ACBP on ACC activity

Hexadecanoyl-CoA proved, as expected, to be a highly potent inhibitor of ACC activity (Figure 4a). In a plot with linear scaled abscissa the IC_{50} value in the control experiment was found to be 0.7 μM when 70 nM ACC was present in the incubation mixture.

In the presence of 1 μM ACBP, the ACC activity remained equal to control until the concentration of hexadecanoyl-CoA had reached 0.8 μM, and the IC_{50} value of hexadecanoyl-CoA inhibition was increased to 1.2 μM. In the presence of 4 μM ACBP the IC_{50} was increased to 5.3 μM (Figure 4a). In the experiments with both 1 and 4 μM ACBP, the activity of ACC was unaffected by the presence of hexadecanoyl-CoA as long as the ratio of hexadecanoyl-CoA to ACBP was below 0.8.

ACBP was also able to restore activity of ACC when it was completely inhibited by 5 μM hexadecanoyl-CoA before addition of ACBP (Figure 4b).

The low K_i (5 nM) for hexadecanoyl-CoA inhibition of ACC activity, and the fact that it has been shown that ACC forms equimolar complexes with hexadecanoyl-CoA even in the presence of phosphatidylcholine (Ogiwara et al., 1978), implies that ACC has a high affinity for long-chain acyl-CoA esters. However, the above results indicate that ACBP has a higher affinity for long-chain acyl-CoA esters than does ACC. This was further illustrated by a binding assay based on separation of the protein/acyl-CoA from free ligand on a 20 cm × 1.25 cm Sephadex G-50 gel-filtration column. ACC was co-incubated with an equimolar amount of radioactive hexadecanoyl-CoA for 30 min at 25 °C and passed over the column. Using this assay we were unable to detect any co-elution of radioactivity with the ACC protein peak (results not shown). We have previously shown a 36 % recovery of hexadecanoyl-CoA in the ACBP peak after passing an hexadecanoyl-CoA/ACBP mixture (1:2) over a 285 cm × 1.25 cm Sephadex G-50 gel-filtration column (Rasmussen et al., 1990). Evidently, ACC has a much lower affinity for long-chain acyl-CoA esters than does ACBP. We failed by all other means to measure the relative affinities of long-

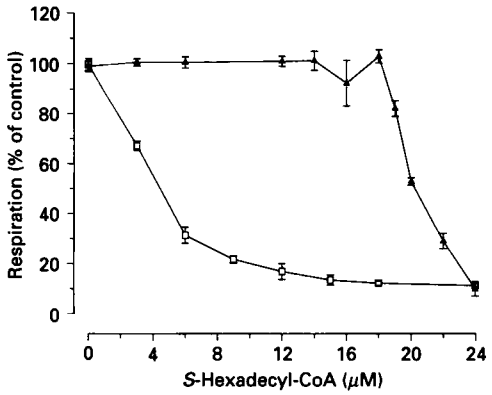

Figure 5 Effects of ACBP on acyl-CoA inhibition of ADP-dependent mitochondrial State-3 respiration

Oxidation of succinate by fed-rat liver mitochondria in the presence of a high concentration of ADP (2 mM) was measured with increasing amounts of *S*-hexadecyl-CoA added in the absence (□) or presence (▲) of 20 μM ACBP. Results are representative of individual experiments and are plotted as mean of duplicates ± half the difference, expressed as percentage of control with no acyl-CoA analogue added. For further details see the Materials and methods section.

chain acyl-CoA esters for ACBP and ACC, due to the large differences in their relative affinities.

Effects of ACBP on ANT

To examine effects of long-chain acyl-CoA esters on ANT, we monitored the State-3 respiration rate in intact rat liver mitochondria (Devaux et al., 1975). In order to avoid hydrolysis of the long-chain acyl-CoA esters during incubation with mitochondria, the non-hydrolysable hexadecanoyl-CoA analogue *S*-hexadecyl-CoA was used. Binding studies have shown that *S*-hexadecyl-CoA binds to ACBP with an affinity similar to that of hexadecanoyl-CoA (Rosendal et al., 1993).

S-Hexadecyl-CoA is an effective inhibitor of the ADP-dependent State-3 respiration (Figure 5). The presence of 20 μM ACBP had a dramatic effect on the *S*-hexadecyl-CoA inhibition of respiration (Figure 5). With a molar ratio of *S*-hexadecyl-CoA to ACBP below 0.8–0.9 the added acyl-CoA analogue did not affect the respiration. ACBP was also able to reverse the inhibition of ANT by the long-chain acyl-CoA ester analogue. When the mitochondria were incubated with *S*-hexadecyl-CoA (24 μM) before addition of ACBP, it was possible to recover 32.54 ± 6.12 % or 63.35 ± 0.7 % ($n = 3$) of the State-3 respiration in the presence of 30 or 60 μM ACBP respectively. The rate of re-activation was relatively slow and showed a maximal effect only after 10 min incubation. This relatively slow rate of re-activation is probably due to the fact that ACBP has no or limited access to the mitochondrial inner membrane, where ANT is located.

Intracellular levels of long-chain acyl-CoA esters and ACBP

Levels of long-chain acyl-CoA esters in fed-rat liver have been reported to be from 15 to 83 nmol/g wet wt. (Bortz and Lynen, 1963; Tubbs and Garland, 1964; Woldegiorgis et al., 1985; Prasad et al., 1987; Panov et al., 1991; Tardi et al., 1992). The reason for the large variations in the reported values might be explained by animal variations, diet composition and/or differences in the methods used.

Table 2 Long-chain acyl-CoA esters and ACBP content in the liver of fed rats

Long-chain acyl-CoA ester and ACBP were analysed as described in the Materials and methods section. The results represent means ± S.D. The cytosolic content of long-chain acyl-CoA esters was calculated by subtracting the mitochondrial pool (14.5%) from the total amount.

	Content (nmol/g wet wt.)
ACBP	53.3 ± 3.9 (6)
Total long-chain acyl-CoA	51.5 ± 2.6 (6)
Cytosolic long-chain acyl-CoA	43.8 ± 2.2

We have used a newly developed method to determine the tissue concentration of long-chain acyl-CoA esters. By using this method we found that the liver of fed rats contained 51.5 ± 2.6 nmol of long-chain acyl-CoA/g of liver (Table 2). Mitochondria were shown to contain 14.5 % of the total long-chain acyl-CoA, corresponding to 0.23 nmol/mg of mitochondrial protein. This is in accordance with the 0.2 nmol/mg of mitochondrial protein reported for epididymal adipocyte mitochondria (Moore et al., 1992).

The ACBP content in the livers used for acyl-CoA determination above was 53.3 ± 3.9 nmol/g of tissue, giving a ratio of long-chain acyl-CoA to ACBP of 0.97 ± 0.09. With 15 % of the long-chain acyl-CoA located in the mitochondria, the cytosolic long-chain acyl-CoA/ACBP ratio is 0.82 ± 0.09. However, the cytosolic pool must be even smaller, because some long-chain acyl-CoA is located in peroxisomes and in membranes, being bound to acyl-CoA metabolizing enzymes. The resulting cytosolic acyl-CoA/ACBP ratio is therefore likely to be lower than 0.82.

Conclusion

The present data strongly indicate that it is ACBP and not FABP which is involved in desorbing long-chain acyl-CoA esters from lc-ACS, and thereby forming an intracellular protected pool of these esters. The results also indicate that long-chain acyl-CoA synthesized by either mitochondrial or microsomal lc-ACS may enter the acyl-CoA/ACBP pool, making long-chain acyl-CoA esters synthesized by both forms of lc-ACS uniformly available in the cell. Furthermore, the data show that lc-ACS needs to be studied in intact membranes in order to obtain the true regulatory properties and activities of the enzyme.

Akerboom et al. (1977) showed that the inhibitory effects of long-chain acyl-CoA esters on mitochondrial nucleotide transport were not seen in intact liver cells. The present results, which clearly demonstrate that ACBP efficiently protects ACC and ANT against the inhibitory effect of long-chain acyl-CoA esters, may explain why the potent inhibitory effects of these esters are not seen *in vivo*. If the acyl-CoA/ACBP ratio is below 1 *in vivo*, long-chain acyl-CoA esters would not be expected to affect nucleotide transport and ACC activity irrespective of the actual level of long-chain acyl-CoA. Thus the ratio of long-chain acyl-CoA esters to ACBP is the key parameter from a regulatory point of view, rather than the level of long-chain acyl-CoA esters.

It is shown that the acyl-CoA/ACBP level in fed-rat liver is below 0.8, and that ACC and ANT therefore will be unaffected by long-chain acyl-CoA esters, although the cytosolic content of acyl-CoA is 44 nmol/g wet wt. It is not known whether cytosolic acyl-CoA can overshoot the ACBP concentration in different metabolic states such as fasting and fasting–refeeding.

The accumulated experimental data point to a dual function of

ACBP. We suggest that ACBP is at the same time able to create a large intracellular pool of acyl-CoA and to keep the free concentration of long-chain acyl-CoAs low. The acyl-CoAs need to be there for specific purposes such as β-oxidation, glycerolipid synthesis, Golgi protein transport and protein acylation, but it should not be seen by acyl-CoA-sensitive enzymes such as ACC, ANT and protein kinase C.

This work was supported by the Danish Natural Science Research Council and the Protein Engineering Research Center. We thank Dr. D. G. Hardie, University of Dundee, U.K., for donation of acetyl-CoA carboxylase, and Dr. Karsten Kristiansen, Odense University, for fruitful discussions. We also thank Erling Knudsen and Rikke R. Sørensen for excellent technical assistance and Bente Meng for typing the manuscript.

REFERENCES

Akerboom, T. P. M., Bookelman, H. and Tager, J. M. (1977) FEBS Lett. **74**, 50–54
Andersen, K. V., Ludvigsen, S., Knudsen, J. and Poulsen, F. M. (1991) Biochemistry **30**, 10654–10663
Berge, R. K. (1979) Biochim. Biophys. Acta **574**, 321–333
Berge, R. K. and Farstad, M. (1979) Eur. J. Biochem. **96**, 393–401
Berge, R. K., Flatmark, T. and Osmundsen, H. (1984) Eur. J. Biochem. **141**, 637–644
Bortz, W. M. and Lynen, F. (1963) Biochem. Z. **339**, 77–82
Burnett, D. A., Lysenko, N., Manning, J. A. and Ockner, R. K. (1979) Gastroenterology **77**, 241–249
Burrier, R. E., Manson, C. R. and Brecher, P. (1987), Biochim. Biophys. Acta **919**, 221–230
Devaux, P. F., Bienvenüe, A., Lauquin, G., Brisson, A. D., Vignais, P. M. and Vignais, P. V. (1975) Biochemistry **14**, 1272–1280
Estabrook, R. W. (1967) Methods Enzymol. **10**, 41–47
Hach, M., Pedersen, S. N., Börchers, T., Højrup, P. and Knudsen, J. (1990) Biochem. J. **271**, 231–236
Hesler, C. B., Olymbios, C. and Haldar, D. (1990) J. Biol. Chem. **265**, 6600–6606
Jamdar, S. C. (1979) Arch. Biochem. Biophys. **195**, 81–94
Knudsen, J. (1976) Comp. Biochem. Physiol. **53B**, 3–7
Knudsen, J. and Nielsen, M. (1990) Biochem. J. **265**, 927–928
Knudsen, J., Højrup, P., Hensen, H. O. and Roepstorff, P. (1989) Biochem. J. **262**, 513–519
Knudsen, J., Mandrup, S., Rasmussen, J. T., Andreasen, P. H., Poulsen, F. and Kristiansen, K. (1993) Mol. Cell. Biochem., in the press
Krisans, S. K., Mortensen, R. M. and Lazarow, P. B. (1980) J. Biol. Chem. **255**, 9599–9607
Lessler, M. A. and Brierley, G. P. (1969) Methods Biochem. Anal. **17**, 1–29
Mandrup, S., Højrup, P., Kristiansen, K. and Knudsen, J. (1991) Biochem. J. **276**, 817–823
Mandrup, S., Hummel, R., Ravn, S., Jensen, G., Andreasen, P., Gregersen, N., Knudsen, J. and Kristiansen, K. (1992) J. Mol. Biol. **228**, 1011–1022
Mandrup, S., Jepsen, R., Skøtt, H., Rosendal, J., Højrup, P., Kristiansen, K. and Knudsen, J. (1993) Biochem. J. **290**, 369–374
Mannaerts, G. P., van Veldhoven, P., van Broekhoven, A., Vanderbroek, G. and Debeer, L. J. (1982) Biochem. J. **204**, 17–23
Mikkelsen, J. and Knudsen, J. (1987) Biochem. J. **248**, 709–714
Mikkelsen, J., Højrup, P., Nielsen, P. F., Roepstorff, P. and Knudsen, J. (1987) Biochem. J. **245**, 857–861
Miyazawa, S., Hashimoto, T. and Yokota, S. (1985) J. Biochem. (Tokyo) **98**, 723–733
Moore, K. H., Dandurand, D. M. and Kiechle, F. L. (1992) Int. J. Biochem. **24**, 809–814
Morand, O. and Aigrot, M. S. (1985) Biochim. Biophys. Acta **835**, 68–76
Nikawa, J., Tanabe, T., Ogiwara, H., Shiba, T. and Numa, S. (1979) FEBS Lett. **102**, 223–226
Ogiwara, H., Tanabe, T., Nikawa, J. and Numa, S. (1978) Eur. J. Biochem. **89**, 33–41
Pande, S. V. (1973) Biochim. Biophys. Acta **306**, 15–20
Panov, A., Solovyov, V. and Vavilin, V. (1991) Int. J. Biochem. **23**, 875–879
Pfanner, N., Orci, L., Glick, B. S., Amherdt, M., Arden, S. R., Malhotra, V. and Rothman, J. E. (1989) Cell **59**, 95–102
Powell, G. L., Tippett, P. S., Kiopes, T. C., McMillin-Wood, J., Coll, K. E., Schultz, H., Tanka, K., Kang, F. S. and Shrago, E. (1985) Fed. Proc. Fed. Am. Soc. Exp. Biol. **44**, 81–84
Prasad, M. R., Sauter, J. and Lands, W. E. M. (1987) Anal. Biochem. **162**, 202–212
Prentki, M., Vischer, S., Glennon, M. C., Regazzi, R., Deeney, J. T. and Corkey, B. E. (1992) J. Biol. Chem. **267**, 5802–5810
Rasmussen, J. T., Börchers, T. and Knudsen, J. (1990) Biochem. J. **265**, 849–855
Reinhart, P. H., Taylor, W. M. and Bygrave, F. L. (1982) Biochem. J. **204**, 731–735
Richards, E. W., Hamm, M. W. and Otto, D. A. (1991) Biochim. Biophys. Acta **1081**, 23–28
Rosendal, J. and Knudsen, J. (1992) Anal. Biochem. **207**, 63–67
Rosendal, J., Ertbjerg, P. and Knudsen, J. (1993) Biochem. J. **290**, 321–326
Schachterle, G. R. and Pollack, R. L. (1973) Anal. Biochem. **51**, 654–655
Shindo, Y., and Hashimoto, T. (1978) J. Biochem. (Tokyo) **84**, 1177–1181
Singh, I., Singh, R., Bhushan, A. and Singh, A. K. (1985) Arch. Biochem. Biophys. **236**, 418–426
Smith, S., Easter, D. J. and Dils, R. (1966) Biochim. Biophys. Acta **125**, 445–455
Suzuki, H., Kawarabayasi, Y., Kondo, J., Abe, T., Nishikawa, K., Kimura, S., Hashimato, T. and Yamamoto, T. (1990) J. Biol. Chem. **265**, 8681–8685
Tanaka, T., Hosaka, K., Hoshimaru, M. and Numa, S. (1979) Eur. J. Biochem. **98**, 165–172
Tardi, P. G., Mukherjee, J. J. and Choy, P. C. (1992) Lipids **27**, 65–67
Tipper, J. P. and Witters, L. A. (1982) J. Biol. Chem. **257**, 162–169
Tubbs, P. K. and Garland, P. B. (1964) Biochem. J. **93**, 550–557
Wise, B. C. and Kuo, J. F. (1983) Biochem. Pharmacol. **32**, 1259–1264
Woldegiorgis, G., Yousufzai, S. Y. K. and Shrago, E. (1982) J. Biol. Chem. **257**, 14783–14787
Woldegiorgis, G., Spennetta, T., Corkey, B. E., Williamson, J. R. and Shrago, E. (1985) Anal. Biochem. **150**, 8–12
Wu-Rideout, M. Y. C., Elson, C. and Shrago, E. (1976) Biochem. Biophys. Res. Commun. **71**, 809–816

Received 1 September 1992/4 February 1993; accepted 16 February 1993