Gene synthesis, expression in *Escherichia coli*, purification and characterization of the recombinant bovine acyl-CoA-binding protein

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A synthetic gene encoding the 86 amino acid residues of mature acyl-CoA-binding protein (ACBP), and the initiating methionine was constructed. The synthetic gene was assembled from eight partially overlapping oligonucleotides. Codon usage and nucleotides surrounding the ATG translation-initiation codon were chosen to allow efficient expression in *Escherichia coli* as well as in yeast. The synthetic gene was inserted into the expression vector pKK223-3 and expressed in *E. coli*. In maximally induced cultures, recombinant ACBP constitutes 12–15% of total cellular protein. A fraction highly enriched for recombinant ACBP was obtained by extracting induced *E. coli* cells with 1 M-acetic acid. Recombinant ACBP was purified to homogeneity by successive use of gel-filtration chromatography, ion-exchange chromatography and reverse-phase h.p.l.c. Recombinant ACBP differed from native ACBP by lacking the *N*-terminal acetyl group. The acyl-CoA-binding characteristics of recombinant ACBP did not differ from those of native ACBP, and the two proteins showed the same ability to induce medium-chain acyl-CoA synthesis by goat mammary-gland fatty acid synthetase. It was concluded that the *N*-terminal acetyl group is not important for acyl-CoA binding.

INTRODUCTION

Acyl-CoA-binding protein (ACBP) was first detected in, and isolated from, bovine liver, on the basis of its ability to induce medium-chain fatty-acid synthesis by goat mammary-gland fatty acid synthetase *in vitro* (Mogensen *et al.*, 1987). The amino acid sequence for bovine and rat ACBP has been determined (Mikkelsen *et al.*, 1987; Knudsen *et al.*, 1989). The sequences of the two proteins were very similar (83% identity). ACBP was found in all tissues tested in cow and rat, with the highest concentration in liver [0.3–0.5% of soluble protein (Mikkelsen & Knudsen, 1987; Knudsen *et al.*, 1989)].

Comparison of the relative binding affinity of ACBP and fatty acid-binding protein (FABP) for long-chain acyl-CoA esters showed that ACBP has much higher affinity for these esters than FABP (Rasmussen *et al.*, 1990). In these studies, only ACBP and not FABP could extract acyl-CoA esters from multilaminar liposomes and prevent binding of these esters to microsomal membranes. The concentration of ACBP in rat liver is 2–4-fold the concentration of long-chain acyl-CoA esters (Knudsen *et al.*, 1989). We therefore concluded that ACBP, and not FABP, is likely to be the physiological carrier of long-chain acyl-CoA esters *in vivo* in the liver.

Simultaneously and independently of the above studies ACBP was isolated from bovine and rat brain on the basis of its claimed ability to displace benzodiazepines from the benzodiazepine-binding site on the γ -aminobutyric acid-receptor complex (GABA_A receptor) (Guidotti *et al.*, 1983). Those authors named the protein diazepam-binding inhibitor (DBI). They suggested that ACBP/DBI was the natural neurotransmitter for the benzodiazepine receptor. However, direct evidence that ACBP/DBI has this function and binds to the benzodiazepine receptor is lacking (Knudsen & Nielsen, 1990). We will therefore

hereafter use the abbreviation ACBP, and not DBI, for this protein.

It has recently been shown that an ACBP variant lacking the C-terminal Gly-Ile can be isolated from bovine adrenals (Besman *et al.*, 1989). This ACBP variant has been shown to stimulate pregnenolone synthesis in bovine adrenal mitochondria.

Further studies are needed to determine whether ACBP, in addition to its ability to bind acyl-CoA, also has a possible neurophysiological function and a specific function in steroidogenesis.

In order to carry out these studies and to obtain sufficient protein to carry out tertiary-structure studies, we decided to make a synthetic gene and express it in *Escherichia coli*. In addition, this would, by the use of site-directed mutagenesis, enable us to synthesize the mutant obtained from bovine adrenals as well as other mutant forms of ACBP likely to be of interest for structural and functional studies. Finally, by choosing a codon usage compatible for efficient expression in yeast, the synthetic gene can be used to study the effect of ACBP on lipid metabolism in yeast.

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase, polynucleotide kinase and *E. coli* DNA polymerase, Klenow fragment and sequencing primers were purchased from New England Biolabs. (Beverly, MA, U.S.A.). Sequenase kit was obtained from USB (Cleveland, OH, U.S.A.), and γ -[³²P]ATP from NEN Research Products (Boston, MA, U.S.A.). Isopropyl β -thiogalactoside (IPTG) was supplied by Clontech (Palo Alto, CA, U.S.A.) and X-gal by Stratagene (La Jolla, CA, U.S.A.). X-ray films were

Abbreviations used: ACBP, acyl-CoA-binding protein; FABP, fatty acid-binding protein; TBST, 50 mM-Tris/HCl (pH 10.0)/150 mM-NaCl/0.5% Tween 20; GABA, γ -aminobutyric acid; DBI, diazepam-binding inhibitor; oligos, oligonucleotides; IPTG, isopropyl β -thiogalactoside; DTE, dithioerythritol; PMSF, phenylmethanesulphonyl fluoride.

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from Fuji (Tokyo, Japan) and fluorescent t.l.c. aluminium sheets and silica-gel 60 F₂₅₄ were from Merck, Darmstadt, Germany. Seakem GTG ('gene technology grade') Agarose and SeaPlaque GTG Agarose were from FMC Bio Products (Rockland, ME, U.S.A.), Elutip-D columns and nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany). Alkaline phosphatase-conjugated swine immunoglobulins against rabbit immunoglobulins were from Dakopatts A/S (Glostrup, Denmark). Trypsin (EC 3.4.21.4) was from Merck, and *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19) was from ICN Biochemicals (Cleveland, OH, U.S.A.). [1-¹⁴C]Acetic acid, [1-¹⁴C]hexadecanoic acid and *cis*-9-[1-¹⁴C]octadecenoic acid were from Amersham International, Amersham, Bucks., U.K. NADPH was from Sigma Chemical Co., St. Louis, U.S.A. Lipidex 1000 was from United Technologies Packard (Downers Grove, IL, U.S.A.). Sephadex G-50 (superfine grade) and Q-Sepharose Fast-Flow were from Pharmacia Biotechnology, Uppsala, Sweden. Nucleosil ODS (10 µm particle size, 10 nm pore size) packing was from Macherey-Nagel, Düren, Germany. Propan-2-ol and trifluoroacetic acid were from Rathburn Chemicals, Walkersburn, Peeblesshire, Scotland, U.K. [1-¹⁴C]-Acetyl-CoA was synthesized enzymically as described previously (Hansen *et al.*, 1984), and [1-¹⁴C]hexadecanoyl-CoA (sp. radioactivity 52 Ci/mol) and *cis*-9-[1-¹⁴C]octadecanoyl-CoA (sp. radioactivity 45 Ci/mol) as described by Rasmussen *et al.* (1990). Malonyl-CoA was synthesized as described by Eggerer & Lynen (1962).

Strains of *E. coli* and plasmids

The bacterial strains *Escherichia coli* DH5α {F⁻, *recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17* (r_k⁻, m_k⁺), *supE44*, *relA1*, λ⁻, Φ80*dlacZ*ΔM15), and XL-1-Blue (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17* (r_k⁻, m_k⁺), *supE44*, *relA1*, λ⁻ (*lac*⁻), [F⁺, *proAB*, *lacI*⁹ΔM15, *Tn10* (tet^R)]} (Bullock *et al.*, 1987) were used as hosts.

The expression vector pKK223-3 was purchased from Pharmacia PL Biochemicals, Uppsala, Sweden.

Construction of the synthetic gene

The ACBP gene was designed so that it could be cloned as four separate fragments each assembled from two oligonucleotides (Fig. 1). The oligonucleotides (oligos) were synthesized by Dr. Lars Hansen, Carlsberg Laboratory, Copenhagen, Denmark. The crude products were gel-purified on 0.9 mm-thick 8 M-urea/15 %-(w/v)-polyacrylamide gels. The oligos were revealed by u.v. shadowing and extracted with 0.2 M-sodium acetate/20 mM-Tris/HCl (pH 7.5)/2 mM-EDTA. The purity and length of the extracted oligos were controlled by labelling with [³²P]ATP and polynucleotide kinase, followed by electrophoresis and exposure to an X-ray film.

Annealing of each pair of oligos was carried out by heating a solution of 20 pmol of each oligo in 50 µl of 50 mM-NaCl/10 mM-Tris/HCl (pH 7.5)/0.1 mM-EDTA to 95 °C, followed by slow cooling in a waterbath to 7 °C below the 'melting' temperature for the overlap. After 1 h at this temperature the mixture was chilled on ice. The solutions were adjusted to 25 mM-NaCl/10 mM-Tris/HCl (pH 7.5)/7 mM-MgCl₂/1 mM-DTE/0.25 mM-dGTP/dATP/dTTP/dCTP, and extension of the strands was done by incubation for 20 min at 45 °C with 20 units of *E. coli* DNA polymerase (large fragment). The ends of the DNA fragments were cut with the appropriate enzymes and inserted in pUC19 vectors. The resulting plasmids were used for transformation of *E. coli* DH5α and sequenced using the dideoxy-chain-termination method. The *acbp* 2 and *acbp* 4 fragments (Fig. 1) were isolated and ligated to pUC19*acbp* 1 and pUC19*acbp* 3 respectively to give pUC19*acbp* 1&2 and pUC19*acbp*

Fig. 1. Construction of the synthetic bovine ACBP gene

(a) The eight oligomers used for the construction after annealing of the four pairs. Restriction sites are indicated. (b) Diagrammatic representation of the ACBP gene. The oligomers are shown as numbered straight lines. Broken lines indicate the parts synthesized with DNA polymerase.

Fig. 2. The pKK223-3 *E. coli* expression vector

The vector contains the strong *tac* promoter (Amann *et al.*, 1983; de Boer *et al.*, 1983a), the *lacZ* ribosome-binding site and the strong transcription terminator from the *E. coli* *rrnB* operon (Brosius *et al.*, 1981).

3&4. The *acbp* 3&4 fragment was isolated and ligated to pUC19*acbp* 1&2 to make pUC19ACBP with an intact ACBP gene.

The *EcoRI*–*HindIII* fragment containing the entire gene was isolated and inserted into the expression vector pKK223-3 (Fig. 2). *E. coli* XL-1-Blue was transformed by pKK223ACBP.

Expression of the synthetic ACBP gene

For a preliminary evaluation of the expression the cells were grown in Luria–Bertani medium containing 50 µg of ampicillin/ml to a density equivalent to an *A*₆₀₀ of 0.5. IPTG (1 mM) was added, and an amount of cells corresponding to 1 ml of an *A*₆₀₀ = 2 solution was sampled at various time points after

induction. The pelleted cells were washed in 0.9% NaCl, resuspended in 70 μ l of 1 mM-phenylmethanesulphonyl fluoride (PMSF) and sonicated for 10 s; 30 μ l of 30 mM-Tris/HCl/1 mM-EDTA (pH 8), 7.5% (w/v) SDS, 15% (v/v) β -mercaptoethanol and 0.03% Bromophenol Blue were added, and the samples were boiled for 3 min. A 1 μ l sample was used for analysis by SDS/PAGE.

For large-scale production the cells were grown in a 4-litre LKB fermenter with automatic pH control. The medium used contained yeast extract (20 g/l), casamino acids (20 g/l), $(\text{NH}_4)_2\text{SO}_4$ (15 g/l), NaH_2PO_4 (2.5 g/l), glucose (10 g/l), thiaminium chloride (10 mg/l) and 5 ml/l of a mineral solution consisting of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (203 g/l), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (2.1 g/l), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.7 g/l), $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (20 mg/l), $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ (10 mg/l), $\text{KCr}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (2 mg/l), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (2 mg/l), H_3BO_3 (1.0 mg/l), KI (20 mg/l), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (20 mg/l), $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ (1 mg/l), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (4 mg/l), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (4 mg/l) and citric acid (21 g/l). IPTG (0.5 mM) was added when the A_{600} was 0.2–0.4, and the cells were grown to an A_{600} of 35–40 in about 10 h. The cells were harvested by centrifugation, washed in 0.9% NaCl and stored frozen at -60°C until purification could be performed.

Purification of native and recombinant bovine ACBP

Native bovine ACBP was purified as previously described (Mikkelsen *et al.*, 1987). For purification of recombinant ACBP, the washed and frozen *E. coli* cells were resuspended in 1 vol. of 2 M-acetic acid and passed through a French press twice. The pH was then adjusted to 7.0 with NaOH and cell debris and precipitated proteins were removed by centrifugation at 12000 g for 20 min. A portion (75 ml) of the supernatant was loaded on to a Sephadex G-50 (superfine grade) column (5 cm $\times$ 80 cm) equilibrated with 30 mM-Tris/HCl, pH 9.0, and eluted with the same buffer at a flow rate of 80 ml/h. The fractions containing ACBP were pooled and loaded on to a Q-Sepharose Fast-Flow column (2.5 cm $\times$ 23 cm) and eluted with a gradient of NaCl in 30 mM-Tris/HCl, pH 9.0, at a flow rate of 4 ml/min. The gradient of buffer B [30 mM-Tris/HCl (pH 9.0)/400 mM-NaCl] in buffer A (30 mM-Tris/HCl, pH 9.0) was as follows: 0–10% for 40 min, 10% constant for 15 min, 10–25% for 60 min and 25–100% for 15 min. The fractions containing ACBP were further purified on a Nucleosil ODS column (8 mm $\times$ 120 mm). Portions (4 ml each) were loaded on to the column equilibrated with 20% solvent B (50% propan-2-ol/0.1% trifluoroacetic acid in water) in solvent A (0.1% trifluoroacetic acid). ACBP was eluted with a gradient of solvent B in solvent A from 20 to 80% solvent B for 43 min at a flow rate of 3 ml/min.

SDS/PAGE

Expression of ACBP in *E. coli* and the purity of the purified ACBP were analysed by electrophoresis through 20% (w/v) homogeneous SDS/polyacrylamide gels using a Pharmacia Phast-Gel apparatus according to the recommendations given by the manufacturer.

The ACBP content in transformed *E. coli* was evaluated by scanning of Coomassie Blue-stained polyacrylamide gels on an LKB Ultrascan XL enhanced laser densitometer.

After termination of electrophoresis the buffer strips were removed and the gel was covered with a wetted nitrocellulose membrane, and the proteins were blotted on to the nitrocellulose by increasing the temperature to 70°C for 1 h.

Immunostaining of Western blots

The nitrocellulose membranes were washed for 3 min in 50 mM-Tris/HCl (pH 10.0)/150 mM-NaCl/0.05% Tween 20 (TBST) and incubated overnight at room temperature in TBST con-

taining 3% BSA. The membranes were washed again for 3 min in TBST and then incubated overnight in TBST containing 3% BSA and 0.28 μ g of rabbit anti-(bovine liver ACBP) antibody/ml. The membranes were then washed for 3 $\times$ 3 min in TBST, followed by washing for 3 min in TBST, pH 7.9, and then incubated for 3 h in TBST, pH 7.9, containing 3% BSA and alkaline phosphatase-conjugated swine immunoglobulins to rabbit immunoglobulins (diluted 1:1000). The membranes were then washed for 3 $\times$ 3 min in TBST, pH 7.9, and for 5 min in 0.1 M-ethanolamine, pH 9.0. The alkaline phosphatase activity was detected by the method of Blake *et al.* (1984).

Sequence analysis

Peptides of recombinant ACBP were obtained by cleavage with trypsin and *Staphylococcus aureus* proteinase as previously described for native bovine (Mikkelsen *et al.*, 1987) and rat (Knudsen *et al.*, 1989) ACBP. Automated Edman degradation was carried out with a Knauer protein sequencer model 810 equipped with an on-line Knauer h.p.l.c. facility for identification and analysis of the phenylthiohydantoin derivatives. The chemicals and program used were as recommended by the manufacturer.

M.s.

The M_r of the intact recombinant ACBP and of all derived peptides was determined on a BioIon BIN 10K plasma-desorption time-of-flight mass spectrometer. The samples were dissolved in 0.1% trifluoroacetic acid, and 10–100 pmol was applied in 2–3 μ l on to an aluminium-treated Mylar foil coated with nitrocellulose and spin-dried (Nielsen *et al.*, 1988). The spectra were recorded for 1×10^6 primary ions.

Incubation with goat-mammary gland fatty-acid synthetase

Goat mammary-gland fatty-acid synthetase (170 μ g; sp. activity 411 nmol of NADPH oxidized/min per mg) was incubated at 37°C in 0.1 M-potassium phosphate buffer (pH 7.0)/1.0 mM-EDTA/240 μ M-NADPH/42 μ M-[1- ^{14}C]acetyl-CoA (sp. radioactivity 10 Ci/mol) in a total volume of 0.5 ml for 15 min. Native and recombinant ACBP were added as indicated. Malonyl-CoA (135 μ M) was infused at a rate of 2 μ l/min. The reaction was stopped by adding 0.5 ml of 5 M-NaOH, and incorporation of radioactivity into individual fatty acids was determined as previously described (Knudsen & Grunnet, 1982).

Acyl-CoA binding to ACBP

The relative binding affinity of native and recombinant ACBP for [1- ^{14}C]hexadecanoyl-CoA (sp. radioactivity 52 Ci/mol) and *cis*-9-[1- ^{14}C]octadecenoyl-CoA (sp. radioactivity 45 Ci/mol) was determined by the Lipidex-1000 assay as previously described (Rasmussen *et al.*, 1990).

RESULTS AND DISCUSSION

Cloning of the synthetic gene

The synthetic gene encoding the 86 amino acid residues of mature bovine ACBP and the initiating methionine residue was designed on the basis of the known amino acid sequence (Mikkelsen *et al.*, 1987). The nucleotide sequence of the synthetic gene was designed by considering the codon usage for highly expressed genes in *Saccharomyces cerevisiae* as well as in *E. coli* (Sharp *et al.*, 1988) to enable efficient expression in both organisms. It has been shown that AT-rich sequences immediately 5' of the initiation codon improve expression of several genes in *E. coli* (de Boer *et al.*, 1983b; Matteucci & Heyneker, 1983). Similarly A-rich sequences are preferred immediately 5' of the initiation codon for highly expressed genes in *Saccharomyces*

Fig. 3. DNA sequence of the synthetic bovine ACBP gene and the amino acid sequence of the recombinant ACBP

Restriction sites are framed and the original synthetic oligomers used for gene synthesis are indicated by continuous lines. The amino acid sequence of the initial 57 amino acids of recombinant ACBP was determined on the intact protein. The remainder of the sequence was verified by sequencing tryptic (T) or *Staphylococcus aureus* proteinase (Sa) peptides as indicated.

cerevisiae (Hamilton *et al.*, 1987), and an A in position -3 has been shown to enhance translation (Baim & Sherman, 1988). Consequently three A residues were inserted 5' of the ATG codon in the synthetic ACBP gene.

When the ACBP gene was cloned in the expression vector pKK223-3 using the *EcoRI* site in the polylinker, the distance from the Shine-Dalgarno sequence to the ATG start codon was 13 bp, which is within the acceptable limits for efficient translation in *E. coli* (Steiz, 1980; Shepard *et al.*, 1982). Three 6 bp restriction-enzyme-recognition sites were placed in the gene to make it possible to clone it as four separate fragments. Excessively stable secondary structures in the encoded mRNA and in the synthetic oligos were avoided. The resulting synthetic ACBP gene is shown in Fig. 3.

Expression of the synthetic ACBP gene

Induction of ACBP synthesis did not alter the growth rate of the XL-1 Blue *E. coli* cells when compared with cells harbouring only the expression vector without insert (results not shown).

SDS/PAGE showed that the ACBP gene was efficiently expressed from pKK223ACBP, and that the ACBP identified by immunostaining of Western blot on nitrocellulose paper tended to reach a stable level at about 4-6 h after IPTG induction (Fig. 4). The maximum level of extractable ACBP was obtained 6 h after induction with IPTG. At this time ACBP made up 6-8% of total protein calculated from scans of Coomassie Blue-stained polyacrylamide gels of whole *E. coli* cells boiled in SDS sample buffer (result not shown). In a large-scale (4-litre) fermenter, ACBP made up 12-15% of total protein. ACBP migrates, as we have shown previously (Mogensen *et al.*, 1987), as a 7.2 kDa, and not a 10 kDa, protein in SDS/PAGE.

The above indicates great stability of the ACBP product and that ACBP is non-toxic to *E. coli*.

The pKK223ACBP plasmid had the following sequence between the Shine-Dalgarno sequence and the ATG start codon:

CAC[AGGA]AACAGAATTCAAAATG

(Shine-Dalgarno sequence in brackets; start codon underlined). As variations in the distance between the ATG start codon and the Shine-Dalgarno sequence have been shown greatly to influence the expression of genes in *E. coli* (Roberts *et al.*, 1979), and since this distance in the case of the pKK223ACBP plasmid was near the upper limit, we deleted 2 bp at the *EcoRI* site. The resulting plasmid, pKK223ACBPΔE1, had the following sequence in this region:

CAC[AGGA]AACAGAACAAAATG

(two T residues deleted at position -5 and -6 relative to the start codon). Incidentally, another clone was found containing the plasmid pKK223ACBPΔE2:

CAC[AGGA]AACAGAATTAATTCAAAATG

(insertion of TTAA between position -6 and -7 relative to the start codon).

SDS/PAGE indicated a significantly higher expression of ACBP with the pKK223ACBPΔE1 plasmid and a significantly lower expression with the pKK223ACBPΔE2 when compared with the original pKK223ACBP plasmid (Fig. 5). Scanning of the Coomassie Blue-stained gels indicated that, in *E. coli*, ACBP expressed from the plasmids pKK223ACBP, pKK223ACBPΔE1 and pKK223ACBPΔE2 made up 6, 12 and 1.4% of the total protein respectively (results not shown).

Extraction of ACBP from *E. coli*

Different methods for the extraction of ACBP from *E. coli* were tried and evaluated by SDS/PAGE. Nearly pure ACBP was extracted from the cells simply by stirring for 20 min in 1 M-acetic acid, but the yield was significantly higher when the cells, during extraction with acetic acid, were sonicated for 4 × 15 s (Fig. 6). However, lane 4/5 (Fig. 6) indicates that the particulate matter from the centrifugations of the acetic acid-extracted cells contained a significant amount of ACBP. The staining of the ACBP band in lane 5 indicates that about 50% of the total ACBP is

Fig. 4. Induction of ACBP expression in *E. coli*

(a) The Figure shows SDS/PAGE of lysates of *E. coli* cells sampled at 0 (lane 1), 1 (lane 2), 2 (lane 3), 4 (lane 4), 6 (lane 5) and 24 (lane 6) h after IPTG induction of ACBP expression; lane 7, 100 ng of native bovine ACBP; lane 8, β -galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (29 kDa), triosephosphate isomerase (26 kDa), myoglobin (17 kDa), cytochrome *c* (12.2 kDa) and aprotinin (6.5 kDa). (b) Immunoblot of a gel similar to that in (a), except that lane 6 contained 20 ng of native bovine ACBP. Lane 8, *E. coli* lysate from cells after overnight induction with IPTG.

present in the particulate fraction. Extending the sonication period to 10×15 s had no significant effect on the amount of ACBP in the particulate fraction.

The high amount of ACBP in the particulate fraction might be due to membrane-associated ACBP. Insoluble aggregates (inclusion bodies), which are often observed with other recombinant proteins expressed at high level in *E. coli* (Williams *et al.*, 1982; Cheng, 1983; Knoerzer *et al.*, 1989), could not be demonstrated. However, as high amounts of ACBP could be recovered in the acetic acid-soluble fraction, we chose to discard the insoluble fraction.

Purification and verification of the structure of recombinant bovine ACBP

After mixing with equal volumes of 2 M-acetic acid and disintegration of the cells in the French press, the ACBP content was evaluated by scanning Coomassie Blue-stained SDS/polyacrylamide gels of the cell homogenate and supernatant after centrifugation. ACBP constituted 23 % of total protein in the homogenate and 53 % of total acid-soluble protein in the supernatant after centrifugation. A simple purification procedure consisting of Sephadex G-50 gel filtration and use of a Q-

Fig. 5. Effect of varying the distance between the Shine-Dalgarno sequence and the start codon

The Figure shows SDS/PAGE of lysates from *E. coli* cells containing different pKK223ACBP vector constructions. Lane 1, pKK223ACBP Δ E1 before induction with IPTG; lanes 2, 3 and 4, pKK223ACBP Δ E1, pKK223ACBP Δ E2 and pKK223ACBP respectively after 6 h induction with IPTG; lane 6 and 7, 20 ng and 100 ng of native bovine ACBP respectively. Lane 8, ovalbumin (43 kDa), carbonic anhydrase (29 kDa), myoglobin (17 kDa), cytochrome *c* (12.2 kDa), aprotinin (6.5 kDa).

Fig. 6. Extraction of ACBP from *E. coli* lysates with acetic acid

The Figure shows SDS/PAGE of lysates of *E. coli* cells: lane 1, before IPTG induction; lane 2, 6 h after IPTG induction (the cells were sonicated in acetic acid, neutralized and cleared by centrifugation); lane 3, the unfractionated lysate; lane 4, the supernatant fraction; lane 5, the pellet fraction; lanes 6, 7 and 8, as in Fig. 5.

Sephacrose fast-flow ion-exchange column gave a homogeneous product (Fig. 7). In order to remove salt and unknown bound ligands, recombinant ACBP was further purified over a reverse-phase column. This step also removed the ACBP dimer present in the Q-Sephacrose Fast-Flow fraction.

Peptides comprising the complete sequence except for the *N*-terminal tetrapeptide were isolated and analysed by m.s. (Table 1), and no modifications could be observed. Native bovine ACBP is *N*-terminally blocked by an acetyl group, but the mass spectrum of the intact protein did not indicate any such group (results not shown).

When approx. 1 nmol of intact recombinant ACBP was subjected to automated sequence analysis, it could be shown that no *N*-terminal blocking group was present, and it was possible to confirm the sequence of the first 57 residues from the *N*-terminal end, except for Trp⁵⁶. The remainder of the sequence was verified by sequencing of peptides obtained by digestion with trypsin or *Staphylococcus aureus* proteinase and aligning these sequences with the previously determined ACBP sequence (Fig. 3). The

Fig. 7. SDS/PAGE of total *E. coli* protein and ACBP fractions during purification

The Figure shows SDS/PAGE of total soluble *E. coli* protein (lane 5), the pooled ACBP peak from the Sephadex G-50 column (lane 4), the pooled ACBP peak from the Q-Sepharose column (lane 3), the pooled ACBP peak from the reverse-phase h.p.l.c. column (lane 2) and the pooled ACBP peak from an overloaded Sephadex G-50 column (lane 1). Molecular-mass markers similar to those in Fig. 5 are shown in lane 6.

Table 1. M.s. determination of M_r values of peptides obtained by digestion of recombinant ACBP with *Staphylococcus aureus* proteinase

For experimental details, see the Materials and methods section. Abbreviation: N.D., not determined.

Peptide	Residue nos.	M_r	
		Calculated	Observed
1	1-4	433.4	N.D.
2	5-11	808.8	808.8
3	12-23	1394.6	1394.5
4	24-60	4361.9	4359.8
5	61-67	761.9	761.9
6	68-79	1411.6	1411.5
7	80-86	849.1	849.2

presence of tryptophan in positions 55 and 58 was verified by m.s. (Table 1). The *N*-terminal methionine residue is observed to have been removed in the recombinant ACBP. Consequently, the lacking *N*-terminal acetyl group is the only detectable difference between recombinant and native ACBP.

Acyl-CoA binding and effect on fatty-acid pattern synthesized by goat mammary-gland fatty acid synthetase by native and recombinant bovine ACBP

It is noteworthy that a small protein like ACBP (M_r 9955) is able to bind acyl-CoA esters with an M_r of 900–1000 (10% of the M_r of the binding protein). This indicates that a large part of the molecule must be involved in forming the binding sites. A small change in primary structure, such as removal of the *N*-terminal acetyl group, might therefore change the binding characteristics. As we had intended to use the recombinant protein to study the tertiary structure and metabolic effect of ACBP, it was important to show that the lacking *N*-terminal acetyl group does not affect binding characteristics.

The apparent K_d for hexadecanoyl-CoA and *cis*-9-octadecenoyl-CoA binding to recombinant bovine ACBP was determined to be $0.17 \pm 0.06 \mu\text{M}$ and $0.14 \pm 0.0 \mu\text{M}$ in six and three individual experiments respectively, using the Lipidex-1000 binding assay. We have previously determined the apparent K_d for hexadecanoyl-CoA and *cis*-9-octadecenoyl-CoA binding to native bovine ACBP to be $0.22 \mu\text{M}$ and $0.14 \mu\text{M}$ respectively (Rasmussen *et al.*, 1990). The binding affinity for long-chain acyl-CoA has therefore not been affected by the lack of the *N*-terminal acetyl group in the recombinant ACBP.

We would like to stress here that the Lipidex-1000 binding assay does not give true K_d values. We have shown that the binding data represent binding equilibrium between ACBP and Lipidex-1000 and not true binding (results not shown). However, for comparison of relative binding affinity the method is acceptable.

To compare further the binding affinity of native and recombinant bovine ACBP for acyl-CoA esters, we compared the ability of the two proteins to induce synthesis of medium-chain acyl-CoA esters by goat mammary-gland fatty acid synthetase. The results (Table 2) show that there is no significant

Table 2. Effect of native (N-ACBP) and recombinant (R-ACBP) bovine ACBP on fatty acid synthesis by goat mammary-gland fatty acid synthetase

Fatty acid synthetase (170 μg ; sp. activity 411 nmol of NADPH oxidized/min per mg) was incubated with the indicated amounts of ACBP. For experimental details, see the Materials and methods section. Values for [^{14}C]acetate incorporated (nmol) are means $\pm$ half the difference between duplicates. Values for the contribution of fatty acid to the total amount of fatty acids synthesized (mol/100 mol) are means of duplicates.

Addition	Amount of ACBP (μg)	Fatty acid...	Pattern of fatty acids synthesized (mol/100 mol)								[^{14}C]Acetate incorporated (nmol)
			C ₄	C ₆	C ₈	C ₁₀	C ₁₂	C ₁₄	C ₁₆	C ₁₈	
Fatty acid synthetase	–		33	11	6	6	12	26	4	2	2.96 $\pm$ 0.02
+ R-ACBP	50		35	11	6	17	17	12	2	–	3.17 $\pm$ 0.18
	100		36	12	9	24	10	8	2	–	3.30 $\pm$ 0.12
	200		37	13	10	26	5	7	2	–	3.68 $\pm$ 0.32
	400		41	16	13	19	4	5	2	–	4.28 $\pm$ 0.01
	800		39	20	15	18	2	4	2	–	4.57 $\pm$ 0.26
+ N-ACBP	50		32	13	7	19	15	12	2	–	3.27 $\pm$ 0.01
	100		36	12	10	24	9	7	2	–	3.21 $\pm$ 0.08
	200		40	15	11	22	5	5	2	–	3.33 $\pm$ 0.09
	400		41	18	13	19	4	4	1	–	3.61 $\pm$ 0.13
	800		45	22	13	14	3	2	1	–	3.95 $\pm$ 0.24

difference in the change in fatty acid pattern induced by native or recombinant ACBP. Preliminary incubation experiments with recombinant ACBP that had only been purified to the Q-Sepharose ion-exchange step showed that recombinant ACBP was less effective than native bovine ACBP in inducing medium-chain fatty acyl-CoA synthesis. However, after the reverse-phase purification step, the two proteins behaved identically. This indicates that an unknown ligand could still be bound to the recombinant ACBP after the ion-exchange step; however, it could also be a salt effect.

Thus all experimental evidence indicates that highly purified native ACBP and recombinant ACBP have identical binding characteristics, and, hence, the recombinant protein is suitable for studies of tertiary structure, metabolic effects and function.

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