Amino acid sequence of acyl-CoA-binding protein from cow liver

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Acyl-CoA-binding protein from bovine liver was purified with the use of reverse-phase h.p.l.c. in the final step. The complete amino acid sequence was determined by using a combination of gas-phase Edman degradation and electron-impact and fast-atom-bombardment mass spectrometry. The sequence was confirmed by determination of the M_r by plasma-desorption time-of-flight mass spectrometry.

INTRODUCTION

We have previously shown that goat mammary-gland fatty acid synthetase has the ability to terminate fatty acid synthesis at medium-chain length by a transacylase function. However, termination by this mechanism can only be accomplished with the aid of an acyl-CoA-removing system. Acyl-CoA binders, such as bovine serum albumin or methylated cyclodextrin, can function as acyl-CoA-removing systems by acting as a sink for the synthesized acyl-CoAs (Knudsen & Grunnet, 1982). In our search for intracellular acyl-CoA binders that could act as carriers of acyl-CoA from the fatty acid synthetase to the triacylglycerol-synthesizing enzymes, we identified an acyl-CoA-binding protein (ACBP) with an apparent M_r of 7000 (Mogensen *et al.*, 1987). This protein could bind and thereby induce medium-chain acyl-CoA synthesis by goat mammary-gland fatty acid synthetase *in vitro*.

In the present paper we further characterize the protein by determining its full amino acid sequence.

MATERIALS AND METHODS

Enzymes and special reagents

Trypsin (EC 3.4.21.4) was from E. Merck, Vydac C₁₈ (10 μ m particle size; 10 nm pore size) packing was from The Separation Group, Hesperia, CA, U.S.A., Nucleosil ODS (10 μ m particle size; 10 nm pore size) packing was from Macherey–Nagel, Düren, Germany, and Spherisorb ODS-2 (10 μ m particle size; 10 nm pore size) was from Phase Separation, Deeside, Queensferry, Clwyd, U.K. *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19) was from Miles Laboratories.

Preparation of G-50 protein

A G-50 fraction, which contained both the fatty acid-binding protein and ACBP, was obtained from bovine liver as described by Haunerland *et al.* (1984), except that a Sephadex G-100 column and a Sephadex G-50 (superfine grade) column were used instead of a Sephacryl S-200 column and a Bio-Gel P-10 column respectively.

Purification of ACBP

The freeze-dried G-50 fraction was dissolved in buffer A [0.1% (v/v) trifluoroacetic acid in water] and

centrifuged at 18000 *g* for 2 min before injection on a h.p.l.c. column (250 mm $\times$ 4 mm) packed with Nucleosil ODS. The column was equilibrated in 20% (v/v) buffer B [0.1% (v/v) trifluoroacetic acid in 50% (v/v) propan-2-ol] in buffer A with a flow rate of 1.0 ml/min. The proteins were eluted with a gradient of buffer B as shown in Fig. 1.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out essentially as described by Laemmli (1970). The M_r markers used were myosin (M_r 200000), α_2 -macroglobulin (M_r 180000), phosphorylase *a* (M_r 94000), bovine serum albumin (M_r 66000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 29000), cytochrome *c* (M_r 11700) and aprotinin (M_r 6500).

Digestion with trypsin

A 0.5 mg portion of ACBP was dissolved in 0.5 ml of 100 mM-Tris/HCl buffer, pH 8.5, containing 10 mM-CaCl₂. Trypsin (50 μ g) was added and the digestion was allowed to proceed at 37 °C for 4 h until the digestion was terminated by injection of the sample on h.p.l.c.

Digestion with *Staphylococcus aureus* proteinase

A 0.5 mg portion of ACBP was dissolved in 0.5 ml of 100 mM-ammonium acetate buffer, pH 4.0. *Staphylococcus aureus* proteinase (50 μ g) was added, and the digestion was allowed to proceed at 37 °C for 18 h until the digestion was terminated by injection on h.p.l.c.

CNBr cleavage

A 0.5 mg portion of ACBP was dissolved in 0.5 ml of 70% (v/v) formic acid, and 50 μ g of CNBr was added. The cleavage was allowed to proceed at room temperature for 20 h and was terminated by injection on h.p.l.c.

Purification of peptides

After cleavage of the protein the peptides were separated on a Vydac C₁₈ column (250 mm $\times$ 4 mm). The column was equilibrated in 4% (v/v) buffer B in buffer A and eluted from the column with a linear gradient of 4–90% (v/v) buffer B in buffer A. The peptides from the tryptic digestion eluted in the solvent peak were freeze-dried and rechromatographed on a Nucleosil ODS column (250 mm $\times$ 4 mm). The column was equilibrated

Abbreviation used: ACBP, acyl-CoA-binding protein.

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Fig. 1. Purification of ACBP by reverse-phase h.p.l.c.

The column (250 mm $\times$ 4 mm) was packed with Nucleosil ODS. The proteins were eluted with a gradient of buffer B in buffer A (----). The ACBP-containing peak is marked with an arrow. For experimental details see the Materials and methods section.

with 5% (v/v) buffer C [0.1% (v/v) trifluoroacetic acid in 40% (v/v) ethanol] in buffer A and the peptides were eluted with a linear gradient of 5–35% (v/v) buffer C in buffer A.

Amino acid analysis

Peptide and protein samples (0.5–2 nmol) were hydrolysed in 6 M-HCl containing 0.1% phenol at 110 °C for 18–24 h in sealed tubes under reduced pressure. After being freeze-dried the hydrolysates were dissolved in 60 mM-sodium citrate buffer, pH 2.20, and portions (one-half to one-twentieth) were loaded on to a Beckman 121MB amino acid analyser equipped with a 0.28 cm $\times$ 16 cm column packed with LA/28/10 (8 μ m particle size) ion-exchange resin (Locarte Co., London, U.K.). The results for the whole protein represent single sample analysis. No corrections were made for hydrolytic loss or incomplete hydrolysis.

Determination of amino acid sequences

Peptides were sequenced on an Applied Biosystems gas-phase Sequenator with chemicals and programme (MHTFA1) as recommended by the manufacturer. A 0.5–2 nmol portion of peptide was used for each sequence run, and the amino acid phenylthiohydantoin derivatives were identified on an HP 1084B instrument with a Spherisorb ODS-2 column (250 mm $\times$ 4 mm) and a gradient of ethanol in 35 mM-sodium acetate buffer, pH 5.2, containing 1% (v/v) tetrahydrofuran (Hunkapiller & Hood, 1983; Esch, 1984).

Mass spectrometry

The M_r of the intact ACBP was determined on a Bio-Ion 10K plasma-desorption time-of-flight mass spectrometer. The sample was dissolved in 5 μ l of water and applied to an aluminium Mylar foil coated with

Fig. 2. SDS/polyacrylamide-gel electrophoresis of purified ACBP

Purified ACBP (10 μ g) was subjected to electrophoresis on an SDS/20% polyacrylamide gel (track 2). M_r markers are shown in track 1.

nitrocellulose (Jonsson *et al.*, 1986). After insertion of the sample in the mass spectrometer it was bombarded with fission fragments from a 10 μ Ci ^{252}Cf source. The spectra were recorded for 5×10^5 primary ions.

Electron-impact mass spectrometry of the blocked *N*-terminal tryptic peptide was performed on a Finnigan MAT 311A mass spectrometer coupled to an SS200 data-acquisition system. The mass spectrometer was operated at a resolution of 1000 and 70 eV electron energy. The sample (approx. 10–15 nmol) was *N*-[$^3\text{H}_3$]acetylated and *NO*-permethylated by using the procedure previously described (Roepstorff, 1978).

The fast-atom-bombardment mass spectrum of the blocked peptide was obtained on the same mass spectrometer as above, but equipped with a saddle-field atom gun (Ion Tech FAB 11 NF). The sample was applied to the tip of the probe in 5 μ l of acetic acid, and 1 μ l of glycerol was added just before complete evaporation of the acetic acid. After insertion in the mass spectrometer the probe was bombarded with 8–9 kV atoms.

RESULTS AND DISCUSSION

Purification of ACBP

ACBP was purified from bovine liver by acid precipitation, heat treatment, Sephadex G-100 gel filtration, Sephadex G-50 gel filtration and reverse-phase h.p.l.c. During the gel filtration ACBP was located in the chromatographic fractions by co-elution with fatty acid-binding protein. Fatty acid-binding protein is easily

Fig. 3. Reverse-phase h.p.l.c. separations of peptides resulting from degradation of bovine ACBP

The peptides were obtained by degradation with (a) CNBr, (b) trypsin and (c) *Staphylococcus aureus* proteinase. All separations were carried out on Vydac C_{18} with a linear gradient of buffer B in buffer A. In (a) the gradient was started 20 min after injection in order to elute the formic acid from the reaction mixture before elution of the peptides.

Fig. 4. Primary structure of bovine ACBP and the peptides used to construct the amino acid sequence

Peptides are numbered from the *N*-terminus. CNBr-cleavage peptides have the prefix CN, tryptic-digest peptides T and *Staphylococcus aureus*-proteinase-digest peptides have the prefix Sa. The numbering is the same as in Fig. 3. Peptides were sequenced either by mass spectrometry (double line) or Edman degradation (heavy line). The thin line indicates peptides checked by amino acid analysis only.

identified because it binds [1^{14}C]oleic acid (Haunerland *et al.*, 1984). The last step in the purification of ACBP was on a reverse-phase h.p.l.c. column as shown in Fig. 1. The ACBP was identified by its ability to induce synthesis of medium-chain fatty acids when incubated with goat fatty acid synthetase as described by Mogensen *et al.* (1987). SDS/polyacrylamide-gel electrophoresis of the purified ACBP (Fig. 2) gave one single band with an apparent M_r of 7000, which is consistent with the value previously published by Mogensen *et al.* (1987).

Sequence determination

When the intact ACBP is subjected to Edman degradation no amino acid phenylthiohydantoin derivative is released, indicating that the *N*-terminus of the protein is blocked. The same observation was made when Edman degradation was attempted on peptides containing the *N*-terminal fragment obtained by degradation of ACBP with CNBr (peptide CN1, Fig. 4), trypsin (peptide T1, Fig. 4) and *Staphylococcus aureus* proteinase (peptide Sa1, Fig. 4). Of these *N*-terminal fragments only peptide T1 was suitable for sequence determination by mass spectrometry, because peptide Sa1 could not be obtained in sufficient quantity. Amino acid analysis of the tryptic peptide showed the composition to be Asx₁Ser₁Glx₂Ala₁Phe₁Lys₁. Fast atom-bombardment mass spectrometry showed the $(M+H)^+$ peak at m/z 866, consistent with the amino acid analysis if one of the acidic residues (Asx, Glx) is in the amidated form and the *N*-terminal blocking group is an acetyl group.

A new preparation of peptide T1 was N -[$^3\text{H}_3$]acetylated and *NO*-permethylated in order to distinguish an already existing acetyl group and acetyl groups introduced during the formation of the derivative. In the spectrum obtained by electron-impact mass spectrometry, peaks were observed at m/z 158, 328 and 413, in agreement

with an assignment of Ac-Ser-Gln-Ala, showing that the acetyl group was not introduced during formation of the derivative. Prominent peaks were also observed at m/z 126, 296 and 381, corresponding to a loss of CH_3OH from the side chain of Ac-Ser. The glutamic acid residue in position 4 was not seen in the mass spectrum. However, evidence that this residue is present in position

Table 1. Amino acid composition of ACBP

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

Amino acid	Composition (mol of residue/mol)	
	Acid hydrolysis	Sequence analysis
Asx	8.7	9*
Thr	3.9	4
Ser	3.0	3
Glx	11.3	12†
Pro	2.0	2
Gly	5.2	5
Ala	8.7	9
Val	3.0	3
Met	2.6	3
Ile	3.7	4
Leu	5.2	5
Tyr	4.0	4
Phe	3.0	3
Lys	15.2	15
His	2.1	2
Arg	1.1	1
Trp	N.D.	2
M_r		9955.3

* Two of which were found in the amidated form.

† Two of which were found in the amidated form.

Fig. 5. Positive-ion plasma-desorption time-of-flight mass spectrum of the molecular-ion region of ACBP

For experimental details see the Materials and methods section.

4 can be derived from the amino acid analysis of peptides T1, CN1 and Sa1, from the M_r of peptide T1 and from the fact that *Staphylococcus aureus* proteinase [which has specificity for the C-terminal side of Asp and Glu (Drapeau, 1977)] cleaves just after this residue. The rest of peptide T1 is overlapped by peptide Sa2.

The separation of the degradation products of ACBP after CNBr cleavage gave four peptides (Fig. 3a), which were expected as ACBP contains three methionine residues (Table 1). The fragments CN2, CN3 and CN4 (Fig. 4) cover the protein from residue 25 onwards to the C-terminal end of the protein, and the CN1 fragment represents the blocked N-terminal end.

Separation of the tryptic peptides of ACBP yielded a more complex picture (Fig. 3b). The peptides eluted in the solvent peak on the Vydac C_{18} column were rechromatographed on a Nucleosil ODS column as described in the Materials and methods section. Peptide T1 was partly sequenced by mass spectrometry as described above. The other peptides were checked by amino acid analysis, and several were sequenced as detailed in Fig. 4. Unexpectedly, trypsin cleaved almost quantitatively after Phe-26 whereas only minor cleavage was obtained after Lys-76. The peak denoted T13 (Fig.

3b) contained a mixture of two peptides where cleavage had occurred after either Lys-82 or Lys-83. The corresponding heterogeneity resulted in the two peaks labelled T12.

The digestion with *Staphylococcus aureus* proteinase showed cleavage after all glutamic acid residues except the three occurrences of -Glu-Glu-, where cleavage occurred only after the second glutamic acid residue. No secondary cleavages were observed.

The amino acid analysis of the intact protein (Table 1) is in very good agreement with the composition calculated from the sequence (Fig. 4). As a further verification of the sequence, the intact protein was analysed by plasma-desorption mass spectrometry (Fig. 5). The spectrum shows a clear signal at m/z 4979.3 assigned as the doubly charged molecular ion MH_2^{2+} and a corresponding weak signal at m/z 9957.2 assigned as MH^+ resulting in a measured M_r of 9956 based on determination of the centroid for the doubly charged ion. Time-of-flight mass spectrometry does not allow unit mass resolution at such high masses. The measured mass is thus the isotopically averaged mass that corresponds within the experimental error ± 5 atomic mass units to the averaged M_r 9955.3 calculated from the sequence shown in Fig. 4.

A computer search of the Protein Identification Resource (May 1986) did not reveal any homology to the sequence determined for ACBP. This confirms that ACBP is a novel protein of a type not previously described. The function of the protein in cow liver is unknown. The only known function of the protein is its ability to induce medium-chain acyl-CoA synthesis by goat mammary-gland fatty acid synthetase *in vitro*.

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