Purification and characterization of variants of acyl-CoA-binding protein in the bovine liver

Mette Skou JENSEN,* Peter HØJRRUP,† Jan Trige RASMUSSEN* and Jens KNUDSEN‡

*Institute of Biochemistry and †Institute of Molecular Biology, Odense University,
Campusvej 55, DK-5230 Odense M, Denmark

INTRODUCTION

Acyl-CoA-binding protein (ACBP) from bovine liver was originally purified and identified by Mogensen et al. (1987). Bovine and rat liver ACBPs have been characterized in detail by Mikkelsen et al. (1987) and Knudsen et al. (1989) respectively. Both proteins are N-acetylated and have 86 amino acids. The average molecular mass values are 9955 and 9932 Da for bovine and rat ACBP respectively. ACBP has recently been shown to be identical to diazepam-binding inhibitor (DBI) (Knudsen & Nielsen, 1990) which has been characterized from a number of species. Comparison of the amino acid sequences of ACBP/DBI between species shows a strong degree of identity (Knudsen, 1991).

When purifying ACBP from bovine liver by ion-exchange chromatography, three ACBP-containing peaks were observed in addition to the main peak. The aim of the present study was to determine the ways in which the ACBP variants found in the minor peaks differed from the N-acetylated ACBP characterized in detail by Mikkelsen et al. (1987).

MATERIALS AND METHODS

Enzymes and special reagents

Bovine livers were obtained from the local slaughterhouse. Sephadex G-50, Sephadex G-100 material and Q-Sepharose-HP column were from Pharmacia, Uppsala, Sweden. Nucleosil ODS (10 µm particle size; 10 nm pore size) and Nucleosil ODS (10 µm particle size; 30 nm pore size) were from Macherey-Nagel, Düren, Germany. Staphylococcus aureus V8 proteinase (EC 3.4.21.19) was from Miles Laboratories. Propan-2-ol (h.p.l.c. grade) and trifluoroacetic acid (gas-phase Sequenator grade) were from Rathburn, Walkernburn, Scotland, U.K. All other chemicals used in this study were of analytical grade and were obtained either from E. Merck or from Ferrak, Berlin, Germany.

Purification of ACBP

ACBP was purified from bovine liver either as described by Hach et al. (1990) or as follows in order to prevent non-enzymic glycosylation. Bovine liver (50–100 g) was homogenized in 3 vol. of 154 mM-KCl and centrifuged at 13000 g for 30 min. The supernatant was further centrifuged at 10^4 g for 60 min. The supernatant was incubated with 1 mmol of [3H]oleic acid (sp. radioactivity 4.8 mCi/mol) for 15 min under gentle stirring and then pumped directly on to a Sephadex G-50 (Superfine grade) column (80 cm × 5 cm). The column was equilibrated and eluted with 30 mM-Tris/HCl (pH 9.0)/0.02% NaN _3 (flow rate 120 ml/h). The eluate was collected in fractions of 12 ml. Radioactivity was determined in 200 µl of each fraction by liquid-scintillation counting. The radioactivity-containing peak representing fatty acid-binding proteins was localized, pooled and loaded on to a Q-Sepharose-HP ion-exchange column (20 cm × 1.6 cm) equilibrated with 30 mM-Tris/HCl, pH 9.0. The proteins were eluted with a gradient of NaN _3 in 30 mM-Tris/HCl, pH 9.0: 0 mM for 5 min, 0 to 40 mM for 40 min, at 40 mM for 15 min, 40 to 100 mM for 60 min and 100 to 400 mM for 5 min. The flow rate was 4 ml/min.

E.l.i.s.a. of ACBP

ACBP was assayed by a multiple-layer enzyme-linked immunochemical procedure essentially as described by Pawlak & Smith (1986), except that reaction of ACBP with immobilized rabbit anti-ACBP was carried out in 10 mM-Tris/HCl (pH 9.0)/0.5 mM-NaCl/2.6 mM-KCl/0.1% BSA/0.02% NaN _3 overnight at 4 °C. The layers, attached sequentially to a 96-well plastic plate, consisted of monospecific rabbit anti-ACBP IgG, ACBP, biotinylated rabbit anti-ACBP IgG, avidin and biotinylated alkaline phosphatase; the chromogenic substrate was p-nitrophenyl phosphate.

Electrophoresis and isoelectric focusing

All electrophoretic and isoelectric focusing analyses were performed on a Pharmacia Phast System with either 20%-(w/v)-acylamide gels or isoelectric focusing gels with Ampholytes for the pH range 3–9. The analyses were carried out as recommended by the manufacturer. The Pharmacia broad pI (EIF3–9) calibration hit was used.

Digestion with V8 proteinase

A 0.5 mg portion of ACBP was dissolved in 0.5 ml of 100 mM-ammonium acetate buffer, pH 4.0; 50 µg of V8 proteinase was added, and the digestion was allowed to proceed for 18 h at 37 °C until terminated by injection of the sample on h.p.l.c.

Abbreviations used: ACBP, acyl-CoA-binding protein; DBI, diazepam-binding inhibitor; p.d.m.s., plasma-desorption time-of-flight mass spectrometry.

† To whom correspondence and reprint requests should be addressed.
Purification of peptides

After cleavage of the protein, the peptides were separated on a 4 mm \times 250 mm Knauer column packed with Nucleosil ODS (10 \mu m particle size; 30 nm pore size). The column was equilibrated with 100% solvent A [0.1% (v/v) trifluoroacetic acid in water], and the peptides were eluted with the following gradient of solvent B [0.1% (v/v) trifluoroacetic acid in 75% (v/v) propan-2-ol] in solvent A: 2% solvent B for 5 min, 2 to 12% solvent B for 10 min, 12 to 32% solvent B for 10 min, 32 to 73% solvent B for 6 min and 37 to 80% solvent B for 5 min; the flow rate was 1.0 ml/min.

Incubation of a peptide with glucose

A 10 nmol portion of peptide was incubated in 200 \mu l of 0.2 M-sodium phosphate buffer (pH 7.4)/0.1 M-glucose/0.02% NaNO_3. The incubation time was either 25 h at 50 °C or 72 h at 37 °C. The samples were lyophilized and the peptides were repurified as described above.

Mass spectrometry

The molecular mass values of the peptides were determined on a BioIon BIN 10K plasma-desorption time-of-flight (p.d.) mass spectrometer. The samples were dissolved in 0.1% (v/v) trifluoroacetic acid, and 2.5 \mu l (corresponding to 20–50 pmol of peptide) was applied on an aluminized Mylar foil target coated with nitrocellulose and spin-dried (Nielsen et al., 1988). After insertion of the sample in the mass spectrometer, it was bombarded with fission fragments from a 10 \mu Ci 26Cl source. The mass spectra of the peptides were recorded for \(5 \times 10^4\) primary ions.

Determination of amino acid sequences

Peptides were sequenced on a Knauer 810 pulsed-liquid sequencer with chemicals and program 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 a Knauer on-line h.p.l.c. 64 using a 250 mm \times 4 mm Lichrosphere 100 C-18 (5 \mu m particle size) column and a gradient of acetonitrile in 50 mM-sodium acetate buffer, pH 5.2, as described by the manufacturer.

RESULTS AND DISCUSSION

The original purification procedure for ACBP developed by Mikkelsen et al. (1987) only contained three chromatography steps: chromatography on Sephadex G-100, followed by a Sephadex G-50 Superfine column and finally a reverse-phase h.p.l.c. step. The purified ACBP showed only a single band on SDS/PAGE (Mikkelsen et al., 1987).

In later experiments the pure ACBP from the reserve-phase column was separated on a Q-Sepharose HP ion-exchange column, and to our surprise the ACBP separated into four peaks (Fig. 1), all of which reacted with monospecific rabbit anti-(bovine ACBP). Q-Sepharose ion-exchange chromatography of the ACBP-containing peak from Sepharose G-50 showed the same result (results not shown), indicating that bovine liver contains more than one type of ACBP or differentially modified ACBP species.

In order to further characterize the additional three ACBP-containing peaks, they were subjected to isoelectric focusing in a pH 3–9 gradient. Peaks II and III contained two major bands focusing at pH 5.9 and 5.25, and peak IV contained in addition a band focusing at pH 4.85. When incubated with hexadecanoyl-CoA prior to focusing, all bands focused at pH 4.55. The fact that all the bands were able to bind hexadecanoyl-CoA further indicates that the three additional peaks from the ion-exchange column represent ACBP variants.

When the pooled fractions from peaks II, III and IV respectively, were subjected to reverse-phase h.p.l.c., they all split up into two major peaks (results not shown). These peaks were further characterized by peptide mapping.

The primary structure of ACBP has been determined (Mikkelsen et al., 1987) and is shown in Fig. 2. The figure also shows the known V8 protease cleavage sites (reacting at pH 4.0), designated S1a etc.

The individual peaks from the h.p.l.c. separations of peaks II, III and IV from the Q-Sepharose column were digested with V8 protease, the individual peptides were isolated by h.p.l.c. and the molecular masses were determined by p.d.m.s. In addition to the well-known peaks in the V8 protease map of ACBP (Fig. 3a), the map of peaks IIb, IIIb and IVb contained an extra peak (marked X in Fig. 3b). The additional peak at the beginning of the chromatogram is an impurity, which also occasionally appears in peptide maps of ACBP at pI 5.9.

The mass determination of the V8 protease peptides showed that the mass of some peptides was different from that expected. A number of peptides from all ACBP variants showed a peak for both the expected molecular mass and the expected mass + 162 Da in p.d.m.s. (results not shown). The mass of peptide X from peaks IIb, IIIb and IVb was 42 Da higher than the expected mass of Sa3 (see Fig. 2). Peptide X was subjected to Edman degradation, and it could be shown to be Sa3 (Val^{13}–Lys^{35}) modified in Lys^{84}. The mass difference of 42 Da

![Fig. 1. Q-Sepharose HP-chromatogram of h.p.l.c.-purified ACBP](image)

ACBP purified as described by Mikkelsen et al. (1987) was rechromatographed on a Q-Sepharose HP ion-exchange column (20 cm x 1.6 cm). The protein was eluted by a gradient of solvent B in solvent A (------). The ACBP-containing peaks are numbered, with peak 1 being native N-acetylated ACBP. For experimental details, see the Materials and methods section.

![Fig. 2. Primary structure of bovine ACBP](image)

V8 protease cleavage sites (pH 4.0) are marked with arrows and V8 protease peptides are shown with the prefix Sa. The peptides are numbered from the N-terminus.
between Sa3 and peptide X strongly indicates that Lys\(^{18}\) is acetylated in the latter. The 162 Da mass increase cannot be explained by exchange of one amino acid for another or by a post-translational modification where an amino acid is attached to the protein. However, non-enzymic glycosylation is a post-translational modification that could give rise to a mass increase of 162 Da. Non-enzymic glycosylation is the reaction where a glucose molecule is bound to a lysine residue through an amide bond. When a peptide which showed the 162 Da mass increase was subjected to Edman degradation, it could be shown that the lysine residues had been modified. However, the modification was never located to one single lysine residue but was evenly distributed over all the lysine residues in the peptide. The normal procedure for purifying ACBP exposes ACBP to rather harsh conditions, including freeze-drying, heat treatment and acid precipitation, and it is possible that the non-enzymic glycosylation arises as a result of these treatments. To verify that ACBP might be non-enzymically glycosylated during purification, we incubated peptide Sa3 from native ACBP with glucose as described in the Materials and methods section. The results showed that it is possible to introduce a mass increase of 162 Da (results not shown). Although no quantitative estimation of the extent of glycosylation was carried out, the ratio of the two m.s. peaks indicated that 2–5% of the peptide had become glycosylated.

To prevent non-enzymic glycosylation during purification, a new and milder method for purifying ACBP was developed. The homogenized tissue was no longer subjected to acid precipitation, heat treatment and freeze-drying, but was loaded directly on to the Sephadex G-50 column after centrifugation. The ACBP-containing fractions were pooled and further purified on a Q-Sepharose HP ion-exchange column. The Q-Sepharose chromatogram now showed three well-separated ACBP-containing
variations were artefacts that arose during the purification method of Hach et al. (1990).

To show that the presence of the two ACBP variants was not an isolated event, liver homogenates from four different animals were examined. The animals were two cows, a bull and a young calf. Purification of ACBP from adult animals showed that both ACBP variants were present. The amount of each variant relative to the amount of the native ACBP pl 5.9 form was similar for all animals. However, when liver from the young calf was examined, only native ACBP could be found.

Neither the acetylation nor the malonylation of ACBP affected its binding affinity for acyl-CoA esters. This question was examined by use of e.p.r. Using this technique we compared the ability of hexadecanoyl-CoA to displace 12-doxoyleic decanoyl-CoA from the three ACBP variants. It was shown that the Lys\textsuperscript{18}-acetylated and -malonated variant has exactly the same affinity for 12-doxoyleic decanoyl-CoA as native ACBP (results not shown). It could therefore be concluded that the binding affinity of ACBP is not altered by modification of Lys\textsuperscript{18}.

Another point which raises the question of whether the modification of Lys\textsuperscript{18} is of general importance in regulating ACBP activity is the fact that this lysine residue is not conserved in all known ACBP forms, as the murine ACBP (Owens et al., 1989) and the rat ACBP (Knudsen et al., 1989) have a glutamine residue in this position. The mechanism of acetylation and malonylation in vivo is unknown; it could be spontaneous, enzymic or part of an unknown enzymic activity exerted by bovine ACBP. The physiological significance, if any, of these modifications in bovine liver ACBP is therefore an open question. In this connection, it is interesting that both the pl 5.25 and pl 4.85 variants are found in bovine brain in the same proportions as in the liver (J. Knudsen, unpublished work).

We thank Lisbeth Johansen for typing the manuscript and Jesper Rosendal for performing the e.p.r. binding studies. The work was financially supported by the Danish Natural Science Research Council with grants to P. H. and J. K.

REFERENCES