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

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Four differently modified forms of acyl-CoA-binding protein (ACBP) were identified in ACBP purified from bovine liver. The majority of the purified ACBP was focused at pH 5.9 in isoelectric focusing and could be shown to be *N*-acetylated ACBP without any further modifications. Two minor peaks were focused at pH 5.25 and 4.85 respectively. Mass spectrometry and sequence determination showed that the pI 5.25 form was acetylated at Lys¹⁸ and that the pI 4.85 form was malonylated in the same position. Furthermore, it could be shown that non-enzymic glycosylation occurred during purification. The acetylated and malonylated variants of ACBP were only found in adult cattle.

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 Machery–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, Walkerburn, 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 10⁵ *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% NaN₃ (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 NaCl in 30 mM-Tris/HCl, pH 9.0: 0 M 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 M-NaCl/2.6 mM-KCl/0.1% BSA/0.02% NaN₃ 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)-acrylamide 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.

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Fig. 3. V8 proteinase-peptide map of h.p.l.c.-purified ACBP variants

The V8 digests of native ACBP (a) and peak IVb (b) were separated on a Nucleosil ODS column as described in the Materials and methods section. The numbering of the peptides refers to Fig. 2. The new peak not found in native ACBP V8 proteinase map is marked X.

between Sa3 and peptide X strongly indicates that Lys¹⁸ 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

Fig. 4. Chromatogram from Q-Sepharose HP ion-exchange separation of the ACBP-containing fractions from Sephadex G-50 chromatography

The modified purification method to prevent non-enzymic glycosylation was used. The ACBP-containing fractions from Sephadex G-50 chromatography were loaded on to a Q-Sepharose HP column, and the proteins were eluted with a gradient of solvent B in solvent A, as shown by the broken line. The ACBP-containing peaks were identified by e.l.i.s.a. and are marked I, II and III. For experimental details, see the Materials and methods section.

Fig. 5. Isoelectric focusing of the ACBP-containing peaks from Fig. 4

Approx. 1 µg of protein from each peak was subjected to isoelectric focusing. Lane 1, peak III; lane 2, peak II; lane 3, peak I; lane 4, pI standards. For experimental details, see the Materials and methods section.

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

Fig. 6. V8 proteinase-peptide map of h.p.l.c.-purified ACBP variants

The V8 proteinase digests of ACBP variant II were separated on a nucleosil ODS column as described in the Materials and methods section. The numbering of the peptides refers to Fig. 2. X indicates the modified peptide.

peaks, of which the major peak again could be shown to be native *N*-acetylated ACBP (Fig. 4).

To clarify whether the ACBP variants seen after the milder purification method were the same as described above, ACBPs from all three ACBP-containing peaks were purified to homogeneity by reverse-phase h.p.l.c. and subjected to isoelectric focusing. The three ACBP variants had different isoelectric points, pI 4.85, pI 5.25 and pI 5.9 (Fig. 5). The variants were digested by V8 proteinase. The peptide map of the pI 5.9 variant was identical with that of native ACBP (Fig. 3a), but the peptide maps of the pI 5.25 variant and the pI 4.85 variant were identical and contained an extra peptide in addition to all of the expected peptides (Fig. 6). When the extra peptides were subjected to Edman degradation both turned out to be Sa3 with Lys¹⁸ modified. The p.d.m.s. analysis of these peptides showed that the peptide of the pI 5.25 variant has a mass increase of 42 Da compared with the mass of native Sa3, whereas the peptide of the pI 4.85 variant has a mass increase of 88 Da. The pI 5.25 variant was identical to the acetylated variant which has already been identified above. A mass increase of 88 Da between the Sa3 peptide and the modified Sa3 peptide in the pI 4.85 variant fits with Lys¹⁸ being malonylated. The shift of pI to 4.85 further supports the presence of a negative group in the molecule attached to Lys¹⁸. We therefore suggest that Lys¹⁸ is malonylated.

In this study no peptides with a mass increase of 162 Da were found, which indicates that the non-enzymically glycosylated

variants 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 pI 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-doxyoctadecanoyl-CoA from the three ACBP variants. It was shown that the Lys¹⁸-acetylated and -malonated variant has exactly the same affinity for 12-doxyoctadecanoyl-CoA as native ACBP (results not shown). It could therefore be concluded that the binding affinity of ACBP is not altered by modification of Lys¹⁸.

Another point which raises the question of whether the modification of Lys¹⁸ 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 pI 5.25 and pI 4.85 variants are found in bovine brain in the same proportions as in the liver (J. Knudsen, unpublished work).

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