Determination by photoaffinity labelling of the hydrophobic part of the binding site for acyl-CoA esters on acyl-CoA-binding protein from bovine liver

Morten HACH,* Søren Nørby PEDERSEN,* Torsten BÖRCHERS,*† Peter HØJRUP† and Jens KNUDSEN*

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

Acyl-CoA esters containing the photoreactive acids 12-(4'-azido-2'-nitrophenoxy)[1-¹⁴C]dodecanoic acid ([¹⁴C]AND-acid) or *N*-(4'-azido-2'-nitro[3',5'-³H]phenyl)-12-aminododecanoic acid ([³H]NANPA-acid) were synthesized. The photoreactive acyl-CoA esters could be bound to bovine acyl-CoA-binding protein (ACBP) and photocrosslinked to the protein. The photocrosslinked acyl-CoA-ACBP complex was separated from unlabelled ACBP on reverse-phase h.p.l.c. and the purified complex was digested with trypsin, *Staphylococcus aureus* V8 proteinase or endoproteinase Asp-N. By four independent peptide maps it was shown that the amino acids taking part in forming the hydrophobic binding site for acyl-CoA esters in bovine ACBP are located on the peptide segment from Asp²¹ to Asp³⁸. Both photoreactive acyl-CoA esters used in this study labelled strongly in the segment from Tyr²⁸ to Ala³⁴. 12-(4'-Azido-2'-nitrophenoxy)[1-¹⁴C]-dodecanoyl-CoA ([¹⁴C]AND-CoA) also introduced a label at position Asp³⁸, but no labelling was found before Ser²⁹. In contrast, *N*-(4'-azido-2'-nitro[3',5'-³H]phenyl)-12-aminododecanoyl-CoA ([³H]NANPA-CoA) also labelled the segment from Asp²¹ to Tyr²⁸. The difference in labelling by the two photoreactive ligands is most likely caused by different mobility of the arylazido group when linked to the fatty acid either through a phenolic *O*- or an anilinic *N*- bond.

INTRODUCTION

Acyl-CoA-binding protein (ACBP) from bovine liver was originally identified and purified by Mogensen *et al.* (1987). The protein has an average *M_r* of 9955 and consists of 86 amino acid residues with an acetylated *N*-terminus (Mikkelsen *et al.*, 1987). ACBP binds medium- and long-chain-length acyl-CoA esters with high affinity (Rasmussen *et al.*, 1990), but is unable to bind fatty acids and CoA alone (Mikkelsen & Knudsen, 1987). The goal of the present study was to identify the part of the sequence involved in forming the hydrophobic domain of the binding site for acyl-CoA esters in bovine ACBP.

MATERIALS AND METHODS

Chemicals, enzymes and special reagents

11-Bromoundecan-1-ol, potassium *tert*-butyrate and 12-aminododecanoic acid were purchased from Aldrich Chemie, Steinheim, Germany. Tributylhexadecylphosphonium bromide was from E. Merck, Darmstadt, Germany. K¹⁴CN and 4-fluoro-3-nitro[2,6-³H]phenylazide ([³H]FNPA) were obtained from Amersham International. 4-Fluoro-3-nitrophenylazide was from Pierce, Rockford, IL, U.S.A. Trypsin (EC 3.4.21.4) was from Sigma Chemical Co., St. Louis, MO, U.S.A. *Staphylococcus aureus* V8 proteinase (SAP; EC 3.4.21.19) was from Miles Laboratories. Endoproteinase Asp-N was from Boehringer, Mannheim, Germany. Sephadex G-50 (superfine), Sephadex G-50 (fine), Q-Sepharose-HP ion-exchange column and CoA 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. Dynosphere PD-102-Re monosized polymeric particles were from Dyno Particles, Lillestrøm, Norway. Propan-2-ol

(h.p.l.c. grade) and trifluoroacetic acid (gas-phase Sequenator grade) were from Rathburn, Walkersburn, Scotland, U.K. All other chemicals used in this study were of analytical grade and were obtained from either E. Merck, or Ferrak, Berlin, Germany.

Purification of ACBP

Bovine ACBP was purified as described by Mikkelsen *et al.* (1987), with the following modifications. The Sephadex G-50 column was run in 30 mM-Tris/HCl, pH 9.0, and fractions containing ACBP from the G-50 column were applied to a 1.6 cm × 10 cm Q-Sepharose HP ion-exchange column equilibrated with 30 mM-Tris/HCl, pH 9.0. ACBP was eluted with a NaCl gradient in 30 mM-Tris/HCl, pH 9.0, from 0–40 mM in 40 min, at 40 mM for 15 min and from 40–100 mM in 60 min. The flow rate was 4 ml/min.

Synthesis of photoreactive acids and their CoA esters

12-(4'-Azido-2'-nitrophenoxy)[1-¹⁴C]dodecanoic acid ([¹⁴C]AND-acid) was synthesized from 11-bromoundecan-1-ol, K¹⁴CN (specific radioactivity 0.212 Ci/mol), tributylhexadecylphosphonium bromide and 4-fluoro-3-nitrophenylazide essentially as described by Moonen *et al.* (1979). *N*-(4'-Azido-2'-nitro[3',5'-³H]phenyl)-12-aminododecanoic acid ([³H]NANPA-acid) was synthesized from 12-aminododecanoic acid and 4'-fluoro-3'-nitro[2',6'-³H]phenylazide (specific radioactivity 8.3 Ci/mol) as described by Jeng & Guillory (1975). CoA esters of the photoreactive acids ([¹⁴C]AND-CoA and [³H]NANPA-CoA) were synthesized as described by Sanchez *et al.* (1973).

Preparation of photocrosslinked acyl-CoA-ACBP complex

Bovine ACBP (200 nmol), dissolved in 0.8–1.0 ml of 0.1 M-potassium phosphate buffer, pH 7.0, was mixed with 300–

Abbreviations used: ACBP, acyl-CoA-binding protein; SAP, *Staphylococcus aureus* proteinase; [¹⁴C]AND-acid, 12-(4'-azido-2'-nitrophenoxy)-[1-¹⁴C]dodecanoic acid; [³H]NANPA-acid, *N*-(4'-azido-2'-nitro[3',5'-³H]phenyl)-12-aminododecanoic acid; [¹⁴C]AND-CoA, 12-(4'-azido-2'-nitrophenoxy)[1-¹⁴C]dodecanoyl-CoA; [³H]NANPA-CoA, *N*-(4'-azido-2'-nitro[3',5'-³H]phenyl)-12-aminododecanoyl-CoA; [³H]FNPA, 4-fluoro-3-nitro[2,6-³H]phenylazide; p.d.m.s., ²⁵²Cf plasma desorption time-of-flight mass spectrometry.

† Present address: Institute of Biochemistry, University of Münster, Wilhelm-Klemmstrasse 2, D-4400 Münster, Federal Republic of Germany.

350 nmol of photoreactive acyl-CoA ester dissolved in 0.5 ml of water. The total volume of the sample did not exceed 1.5 ml. The sample was incubated for 45 min at 37 °C to obtain a complex of ACBP and the photoreactive acyl-CoA.

The complex was separated from unbound photoreactive acyl-CoA ester by gel filtration on a Sephadex G-25 column. The column (18 cm × 0.5 cm) was equilibrated and eluted with 10 mM-potassium buffer (pH 7.3)/0.02 % NaN₃ (flow rate 0.5 ml/min). The eluate was collected in fractions of 1 ml. Radioactivity was determined in 20 µl (¹⁴C label) or in 5 µl (³H label) of each fraction by liquid scintillation counting.

Photolysis was carried out in a 4 ml quartz cuvette, placed directly under the u.v. lamp (Original Hanau, NN 30/38, 35 W) at a distance of less than 5 cm. All operations with photoreactive material before photolysis were carried out in dim light.

Purification of photocrosslinked acyl-CoA-ACBP complex

Photocrosslinked acyl-CoA-ACBP complex was separated from unlabelled ACBP on an h.p.l.c. column (120 mm × 8 mm) packed with Nucleosil ODS (10 µm particle size, 100 nm pore size). The u.v.-irradiated sample (3–4 ml) was injected directly on the h.p.l.c. column equilibrated with 20 % (v/v) solvent B [0.1 % (v/v) trifluoroacetic acid in 50 % (v/v) propan-2-ol] in solvent A [0.1 % (v/v) trifluoroacetic acid in water]. The column was eluted with a gradient of solvent B, with a 0.7 % increase per min up to 80 % (v/v) solvent B in solvent A, with a flow rate of 3.0 ml/min.

Collected fractions were freeze-dried and redissolved in 0.5 ml of water. Radioactivity was determined in 20 µl (¹⁴C label) or 5 µl (³H label) of each fraction by liquid scintillation counting. Fractions containing the photocrosslinked complex, as indicated by radioactivity, were pooled and quantified by liquid scintillation counting.

Digestion with trypsin

Photocrosslinked acyl-CoA-ACBP complex (0.14 mg) was dissolved in 0.1 ml of 0.1 M-ammonium bicarbonate buffer, pH 8.0; 7 µg of trypsin was added and the digestion was allowed to proceed for 4 h at 37 °C. The reaction was terminated by injection of the sample on h.p.l.c.

Digestion with SAP

Photocrosslinked acyl-CoA-ACBP complex (0.1–0.15 mg) was dissolved in 0.1 ml of 0.1 M-ammonium acetate buffer, pH 4.0; 15 µg of SAP was added and the digestion was allowed to proceed for 18 h at 37 °C. The reaction was terminated by injection of the sample on h.p.l.c.

Digestion with endoproteinase Asp-N

Photocrosslinked acyl-CoA-ACBP complex (0.1 mg) was dissolved in 0.1 ml of 50 mM-Na₂HPO₄ buffer, pH 8.0. The enzyme (2 µg), dissolved in 50 µl of water, was added, and digestion was allowed to proceed for 23 h at 37 °C; the reaction was terminated by injection of the sample on h.p.l.c.

Purification of peptides

Tryptic peptides were separated on a 120 mm × 4 mm Knauer column packed with Nucleosil ODS (10 µm particles, 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 a gradient of solvent B [0.1 % (v/v) trifluoroacetic acid in 75 % (v/v) propan-2-ol] in solvent A [0 % solvent B for 2 min, from 0 to 2 % solvent B for 5 min, 2 to 7 % solvent B for 5 min, 7 to 15 % solvent B for 5 min, 15 to 35 % solvent B for 10 min, 35 to 60 % solvent B for 10 min and 60 to 80 % solvent B for 2 min, followed by 80 % (v/v) solvent B in solvent A for 2 min; flow rate 1.0 ml/min].

SAP peptides were separated on 60 mm × 4 mm or 40 mm × 4 mm Knauer columns packed with Dynosphere PD-102-RE monosized particles. The column was equilibrated with 100 % solvent A and the peptides were eluted with a gradient of solvent B in solvent A (from 0 to 2 % solvent B for 2 min, 2 to 12 % solvent B for 10 min, 12 to 32 % solvent B for 10 min, 32 to 37 % solvent B for 5 min and 37 to 80 % solvent B for 5 min, followed by 80 % solvent B in solvent A for 2 min; flow rate 1 ml/min).

Endoproteinase Asp-N peptides were separated on a 40 mm × 4 mm Knauer column packed with Dynosphere PD-102-RE monosized particles. The column was equilibrated with 100 % solvent A (flow rate 1 ml/min) and eluted with a gradient of solvent B in solvent A as described above for the SAP peptides, with the modification that the step from 37 to 80 % solvent B was run for 10 min. Individual peptides were collected, freeze-dried and stored at –20 °C until use.

Peptides were redissolved in 100 µl of solvent A. The radioactivity of the peptides was determined in 5 µl of each sample by liquid scintillation counting. Peptides were further characterized and identified by ²⁵²Cf plasma desorption time-of-flight mass spectrometry (p.d.m.s.) and amino acid analysis.

Mass spectrometry (m.s.)

The *M_r* values of the peptides were determined on a Bio-Ion BIN 10K plasma desorption time-of-flight mass spectrometer. The samples were dissolved in 0.1 % (v/v) trifluoroacetic acid, and 20–50 pmol of peptide was applied in 2.5 µl to 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 µCi ²⁵²Cf source. The mass spectra of the peptides were recorded for 5 × 10⁵ primary ions.

Amino acid analysis

Peptide samples (0.1–0.5 nmol) were hydrolysed in the gas phase by 6 M-HCl/0.1 % phenol at 110 °C for 18–20 h in closed containers under reduced pressure. After freeze drying, the samples were redissolved in 40 µl of 0.01 M-HCl. Portions of 25 µl were used for amino acid analysis on a 250 mm × 4 mm Shandon Hypersil 3 µm h.p.l.c. column with precolumn *O*-phthalaldehyde derivative formation and detected by fluorescence.

Determination of amino acid sequences

Radiolabelled peptides (0.5–1.0 nmol) were sequenced on a Knauer 810 pulsed-liquid sequencer with chemicals and program as recommended by the manufacturer. Samples of 20 µl of the amino acid phenylthiohydantoin derivatives were used for amino acid identification on a Knauer on-line h.p.l.c. 64 using a 250 mm × 4 mm Lichrosphere 100 C-18 (5 µm particle size) column and a gradient of acetonitrile in 50 mM-sodium acetate buffer, pH 5.2, as described by the manufacturers.

The remainder of the amino acid phenylthiohydantoin derivatives (130 µl) were collected and radioactivity was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Synthesis of photoreactive acyl-CoA esters

The synthesis of [¹⁴C]AND-acid resulted in a low yield (10 %), and only 39 µmol of the acid was available for CoA ester synthesis. CoA ester synthesis from 5 µmol of acid, using the method described by Sanchez *et al.* (1973), gave a yield of 24 %. The synthesis of [³H]NANPA-acid gave a yield of 31 %, and CoA ester synthesis from 3.75 µmol of [³H]NANPA-acid gave a

Fig. 1. Chemical structures of photoreactive acyl-CoA esters

(a) [^{14}C]AND-CoA. The carbon atom marked with the arrow contains the ^{14}C label. (b) [^3H]NANPA-CoA. Positions marked with arrows contain the ^3H label.

yield of 47%. The chemical structures of [^{14}C]AND-CoA and [^3H]NANPA-CoA are shown in Fig. 1.

Preparation of photocrosslinked acyl-CoA-ACBP complex

After incubation with [^{14}C]AND-CoA or [^3H]NANPA-CoA, photoreactive acyl-CoA-ACBP complexes could be separated from unbound photoreactive acyl-CoA esters by Sephadex G-25 gel filtration (Fig. 2). Based on the radioactivity in the fractions containing the photoreactive acyl-CoA-ACBP complex, it was calculated that photoreactive acyl-CoA bound to ACBP in a molar ratio of 1:1. Removal of unbound photoreactive ligand before photolysis diminishes the possibility for unspecific labelling by the photoreactive acyl-CoA ester, because the ligand will only be located at the high-affinity binding site in the purified complex. The purified complex was irradiated with u.v. light to form the photocrosslinked acyl-CoA-ACBP complex.

Photocrosslinked acyl-CoA-ACBP could be separated from non-crosslinked ACBP on a reverse-phase h.p.l.c. column (Fig. 3). Based on radioactivity in the purified photocrosslinked ACBP peak, it was calculated that the yield in the photocrosslinking

Fig. 2. Separation of photoreactive acyl-CoA-ACBP complex from unbound ligand

ACBP (200 nmol) was incubated with 250 nmol of [^3H]NANPA-CoA in 1.5 ml of 0.1 M-potassium phosphate buffer, pH 7.4, and separated on a Sephadex G-25 column as described in the Materials and methods section. Fractions of 1.0 ml were collected and radioactivity was determined. Void volume peak is shown by an arrow.

Fig. 3. Separation of photocrosslinked acyl-CoA-ACBP complex from unlabelled ACBP by reverse-phase h.p.l.c.

The reaction mixture from photocrosslinking photoreactive acyl-CoA-ACBP complex was separated on a Nucleosil ODS column as described in the Materials and methods section. The gradient of solvent B in solvent A is shown by the broken line. The peak containing photocrosslinked complex is marked with an arrow. The bars indicate the radioactivity (d.p.m.) in the peaks.

reaction was between 5 and 13% in four different experiments. This yield is in agreement with the results obtained by Moonen *et al.* (1979) using a nitroarylazide derivative as the photoreactive group.

Identification of the binding site

Digestion of bovine ACBP with trypsin and SAP results in 13 and seven peptides respectively (Mikkelsen *et al.*, 1987). A tryptic peptide map of ACBP photocrosslinked to [^{14}C]AND-CoA and separated on a Nucleosil ODS 300 C_{18} 10 μm column is shown in Fig. 4. The peak marked with an arrow contained 33% of the radioactivity applied to the column; the remaining radioactivity could not be detected anywhere in the peptide map. Peptides were characterized by p.d.m.s. and amino acid analysis. The labelled peptides were not amenable to analysis by p.d.m.s. and were therefore further characterized by automatic sequencing. Sequence analysis showed that the radioactive peak contained two peptides, T_4 (17-32) and T_5 (33-50) (Table 1). Numbers refer to positions in the primary sequence of bovine ACBP (Mikkelsen *et al.*, 1987). The amino acid residues that had reacted with the arylazido group in the [^{14}C]AND-CoA ester could not be identified with certainty, because the sample contained two peptides, but the following steps were found to contain radioactivity: Thr¹⁷/Gln³³, Lys¹⁸/Ala³⁴, Ala²⁰/Val³⁶, Glu²²/Asp³⁸, Ser²⁹/Gly⁴⁵, His³⁰/Met⁴⁶ and Lys³²/Asp⁴⁸. Attempts to separate the two peptides were unsuccessful, as the retention of the peptides on reverse-phase h.p.l.c. was determined solely by the large acyl or acyl-CoA group and not by the peptide part. The most likely explanation for the labelling pattern observed above is that labelling has occurred on the C-terminal part of T_4 and the N-terminal part of T_5 . If this is the case, the amino acid residues containing the label will then be Ser²⁹, His³⁰ and Lys³² in T_4 , and

Fig. 4. Tryptic peptide map of photocrosslinked [^{14}C]AND-CoA-ACBP complex

The tryptic digest of photocrosslinked [^{14}C]AND-CoA-ACBP complex was separated on a Nucleosil ODS column as described in the Materials and methods section. The peak containing radioactivity is marked with an arrow. The bars indicate radioactivity (d.p.m.) in the peaks.

Table 1. Sequence analysis of the radioactivity-containing peak in the tryptic map of photocrosslinked [^{14}C]AND-CoA-ACBP complex shown in Fig. 4

For further details, see the Materials and methods section.

Step	Amino acid	Position	Radioactivity counted (d.p.m.)
1	Thr/Gln	17/33	11.5
2	Lys/Ala	18/34	14.6
3	Pro/Thr	19/35	0
4	Ala/Val	20/36	7.2
5	Asp/Gly	21/37	0
6	Glu/Asp	22/38	6.8
7	Glu/Ile	23/39	0
8	Met/Asn	24/40	0
9	Leu/Thr	25/41	0
10	Phe/Glu	26/42	0
11	Ile/Arg	27/43	0
12	Tyr/Pro	28/44	0
13	Ser/Gly	29/45	4.7
14	His/Met	30/46	17.0
15	Tyr/Leu	31/47	0
16	Lys/Asp	32/48	5.4

Gln³³, Ala³⁴, Val³⁶ and Asp³⁸ in T₅. To confirm this interpretation it was necessary to obtain a peptide that covers the whole region of T₄ and T₅ (positions 17–50). SAP peptide Sa4(24–60) partly fulfils this requirement, as only the seven *N*-terminal residues of T₄ are missing. In the peptide map of SAP-digested photocrosslinked [^{14}C]AND-CoA complex on the Nucleosil ODS 300 C₁₈ 10 μm column, no radioactive peak could be observed, and the peak containing peptide Sa4(24–60) was missing (results not shown).

In order to obtain better recovery of the hydrophobic Sa4(24–60) peptide photocrosslinked with [^{14}C]AND-CoA, the SAP peptide map was repeated on an h.p.l.c. column packed with Dynosphere PD-102-RE packing (Fig. 5). The peak marked with an arrow contains 12% of the radioactivity originally applied to the column, and no trace of the normal Sa4(24–60)

Fig. 5. SAP peptide map of photocrosslinked [^{14}C]AND-CoA-ACBP complex

The SAP digest of photocrosslinked [^{14}C]AND-CoA-ACBP complex was separated on a Dynosphere PD-102-RE column (60 mm $\times$ 4 mm) as described in the Materials and methods section. The peak containing the radioactive peptide is marked with an arrow. The bars indicate the radioactivity (d.p.m.) in the peaks.

Table 2. Sequence analysis of the radioactivity-containing peak in the SAP map of the photocrosslinked [^{14}C]AND-CoA-ACBP complex shown in Fig. 5

For further details, see the Materials and methods section.

Step	Amino acid	Position	Radioactivity counted (d.p.m.)
1	Met	24	0
2	Leu	25	0
3	Phe	26	0.7
4	Ile	27	0
5	Tyr	28	1.0
6	Ser	29	2.3
7	His	30	4.6
8	Tyr	31	0
9	Lys	32	0
10	Gln	33	9.2
11	Ala	34	0.6
12	Thr	35	0.7
13	Val	36	2.0
14	Gly	37	0
15	Asp	38	4.9
16	Ile	39	0.5
17	Asn	40	0
18	Thr	41	0
19	Glu	42	0.7
20	Arg	43	0.8

peptide could be detected. The remaining radioactivity could not be located anywhere in the peptide map. Except for the labelled peptide, the remaining SAP peptides in the peptide map were identified by p.d.m.s. and amino acid analysis, and non contained radioactivity. Sequence analysis of the labelled peptide showed that the first 20 amino acid residues in this peptide were identical with the first 20 amino acid residues in the sequence of Sa4(24–60) (Table 2).

The labelling pattern on the Sa4(24–60) peptide was identified as Ser²⁹, His³⁰, Gln³³, Ala³⁴, Val³⁶ and Asp³⁸. This is in agreement with the above suggestion that the part of the sequence containing

the hydrophobic binding site is Ser²⁹-His-Tyr-Lys-Gln-Ala-Thr-Val-Gly-Asp³⁸.

There are, however, two main objections to the labelling experiment described above: (1) the very low number of counts in the fractions from the sequencer, caused by the low specific radioactivity of the [¹⁴C]AND-CoA ligand (0.212 Ci/mol), results in only His³⁰ and Gln³³ showing counts significantly above the background; and (2) the very low recovery of radioactivity in the peptide maps, especially of peptide Sa4(24–60). We tried to solve the first of these problems by using a photoreactive ligand with a higher specific radioactivity. For that purpose we synthesized [³H]NANPA-CoA with a specific radioactivity of 8.3 Ci/mol. To get better recovery of the labelled peptides we separated the SAP peptide map on a 40 mm instead of a 60 mm Dynosphere column. [³H]NANPA-CoA also bound to bovine ACBP with high affinity; the photoreactive acyl-CoA-ACBP complex could be separated from unbound ligand and could be photocrosslinked. The yield of photocrosslinked complex in to independent experiments was about 10%. Photocrosslinked complex was digested with SAP and SAP peptide mapping was carried out using the short Dynosphere column (Fig. 6). The recovery of radioactivity loaded on this column was 48%; the rest of the radioactivity was lost.

Sequence analysis of the peptide in the only radioactivity-containing peak showed unexpectedly that this peak contained, besides peptide Sa4(24–60), a peptide consisting of Sa3+Sa4(12–60) (Table 3). The SAP cleavage site between Sa3(12–23) and Sa4(24–60) appeared to be blocked because continued SAP digestion of the reaction mixture did not change the ratio of the two peptides Sa4(24–60) and Sa3+Sa4(12–60). Also, it was not possible to separate the two peptides on the 4 mm × 40 mm Dynosphere column. Based on the areas of the phenylhydantoin amino acids from sequence analysis, the ratio between the two peptides was about 1:1. The inability of SAP to cleave the peptide bond between Glu²³ and Met²⁴ indicates that photocrosslinking has occurred in this region, suggesting that [³H]NANPA-CoA is labelled differently from [¹⁴C]AND-CoA. This is confirmed by the labelling pattern.

In the former labelling experiments with [¹⁴C]AND-CoA, we did not observe any labelled amino acid prior to position 29 (Ser²⁹). The experiment described above shows a much more widespread labelling. The sequence steps containing highest

Table 3. Sequence analysis of the radioactivity-containing peak from the SAP map of the photocrosslinked [³H]NANPA-CoA-ACBP complex shown in Fig. 6

For further details, see the Materials and methods section.

Step	Amino acid	Position	Radioactivity counted (d.p.m.)
1	Val/Met	12/24	115.7
2	Lys/Leu	13/25	43.6
3	His/Phe	14/26	43.0
4	Leu/Ile	15/27	9.8
5	Lys/Tyr	16/28	102.5
6	Thr/Ser	17/29	45.3
7	Lys/His	18/30	231.8
8	Pro/Tyr	19/31	10.2
9	Ala/Lys	20/32	19.6
10	Asp/Gln	21/33	10.0
11	Glu/Ala	22/34	56.2
12	Glu/Thr	23/35	35.1
13	Met/Val	24/36	90.9
14	Leu/Gly	25/37	0
15	Phe/Asp	26/38	35.2
16	Ile/Ile	27/39	65.1
17	Tyr/Asn	28/40	0
18	Ser/Thr	29/41	253.6
19	His/Gln	30/42	0
20	Tyr/Arg	31/43	9.9
21	Lys/Pro	32/44	0

radioactivity could be identified as Val¹²/Met²⁴ and Lys¹⁸/His³⁰, but Lys¹³/Leu²⁵, His¹⁴/Phe²⁶, Lys¹⁶/Tyr²⁸, Thr¹⁷/Ser²⁹, Glu²²/Ala³⁴, Met²⁴/Val³⁶ and Ser²⁹/Thr⁴¹ also contained significant radioactivity (Table 3). The high content of radioactivity in Val¹²/Met²⁴ and Met²⁴/Val³⁶ indicates that labelling has occurred at Met²⁴ and not at Val¹². The results in Table 3 indicate that labelling could have occurred at both Glu²² and Met²⁴. The high amount of labelling in steps 15, 16 and 18 (Phe²⁶/Asp³⁸, Ile²⁷/Ile³⁹ and Ser²⁹/Thr⁴¹) indicates that Phe²⁶, Ile²⁷ and Ser²⁹ are labelled. This is also in accordance with the labelling of His³⁰ by [¹⁴C]AND-CoA, and it is therefore unlikely that labelling has occurred before Glu²².

In an attempt to identify which amino acid residues before position 23 in the sequence had become photocrosslinked to the [³H]NANPA-CoA ester, we carried out a new experiment and digested the photocrosslinked complex with endoproteinase Asp-N. This proteinase should under ideal conditions yield a peptide that covers the region from Asp²¹ to Gly³⁷. Endoproteinase Asp-N cleavage before Asp²¹ could also show whether or not there had been labelling in the Val¹²-Ala²⁰ region.

Endoproteinase Asp-N peptide mapping was also carried out on the 40 mm × 4 mm Dynosphere column (Fig. 7). About 20% of the radioactivity applied on the column could be recovered in a single peak. Sequence analysis showed that this radioactive peak contained three peptides, one major peptide starting at Asp⁶ and two minor peptides starting at Asp²¹ and Asp⁴⁸ (Table 4). The molar proportions of the peptides were 10:2:1, based on the recovery from the sequencer, and the fact that sequence analysis of the two minor peptides stopped after Thr³⁵ and Ala⁵⁷ respectively. The labelling pattern from step 16 onwards therefore only represents the major peptide from Asp²¹ onwards. The labelling pattern in this area confirms the result from the previous experiment with [³H]NANPA-CoA, which indicated that Met²⁴ and the sequence from Phe²⁶ to Ala³⁴ become labelled. The result also shows that Asp²¹, Glu²² and Glu²³ also become labelled, which explains the blocking of the cleavage of the major peptide at Asp²¹ by endoproteinase Asp-N. In the experiment presented

Fig. 6. SAP peptide map of photocrosslinked [³H]NANPA-CoA-ACBP complex

The SAP digest of photocrosslinked [³H]NANPA-CoA-ACBP complex was separated on a Dynosphere PD-102-RE column (40 mm × 4 mm) as described in the Materials and methods section. The peak containing radioactivity is marked with an arrow. The bars indicate the radioactivity (d.p.m.) in the peaks.

Fig. 7. Endoproteinase Asp-N peptide map of photocrosslinked $[^3\text{H}]\text{NANPA-CoA-ACBP}$ complex

The endoproteinase Asp-N digest of photocrosslinked $[^3\text{H}]\text{NANPA-CoA-ACBP}$ complex was separated on a Dynoshere PD-102-RE column (40 mm $\times$ 4 mm) as described in the Materials and methods section. The peak containing the radioactive peptide is marked with an arrow. The bars indicate the radioactivity (d.p.m.) in the peaks.

in Table 3 there was no labelling of the sequence from Asp⁶ to Glu¹¹. The radioactivity in steps 1–6 in Table 4 must therefore originate from the minor peptide starting at Asp²¹. The labelling in steps 7–12 (Table 4) is more difficult to interpret. The slightly higher labelling in step 9 is most likely caused by Ser²⁹ in the minor sequence, because step 24 (Ser²⁹) in the major peptide is also labelled. The labelling in some of these other steps in this part of the sequence is most likely caused by some memory effect of the system; the slow decrease of labelling in steps 9–12 strongly indicates this. The unexpected high labelling in steps 1–3 is most probably due to unspecific release of radioactivity from the peptides caused by exposure to a high trifluoroacetic acid concentration in the sequencer. The high labelling in steps 13–15 is in accordance with the labelling in Ala³⁴ found in step 29.

We therefore conclude that $[^3\text{H}]\text{NANPA-CoA}$ photolabels ACBP in the sequence covering Asp²¹–Ala³⁴. This overlaps the sequence labelled by $[^{14}\text{C}]\text{AND-CoA}$, from Ser²⁹ to Asp³⁸. The strong labelling from Ser²⁹ to Lys³² by both ligands indicates that this part of the sequence plays an important role in forming the hydrophobic part of the binding site in ACBP. Two-dimensional n.m.r. studies show that this sequence is part of an α -helical structure going from Glu²² to Gly³⁷ (J. Knudsen, unpublished work). The different labelling by the two ligands is most likely due to different mobility of the arylazido group being bound either through an *O*- or *N*-link to the acyl chain of the acyl-CoA.

This work was financially supported by the Carlsberg Foundation and the Natural Science Research Council. We thank Erling Knudsen for

Table 4. Sequence analysis of the radioactivity-containing peak from the endoproteinase ASP-N map of the photocrosslinked $[^3\text{H}]\text{NANPA-CoA-ACBP}$ complex shown in Fig. 7

For further details, see the Materials and methods section.

Step	Amino acid	Position	Radioactivity counted (d.p.m.)
1	Asp/Asp/Asp	6/21/48	218.0
2	Lys/Glu/Phe	7/22/49	166.9
3	Ala/Glu/Lys	8/23/50	194.7
4	Ala/Met/Gly	9/24/51	42.2
5	Glu/Leu/Lys	10/25/52	32.9
6	Glu/Phe/Ala	11/26/53	25.4
7	Val/Ile/Lys	12/27/54	36.0
8	Lys/Tyr/-	13/28/55	23.5
9	His/Ser/Asp	14/29/56	34.4
10	Leu/His/Ala	15/30/57	18.6
11	Lys/Tyr/-	16/31/-	9.6
12	Thr/Lys/-	17/32/-	0.3
13	Lys/Gln/-	18/33/-	38.9
14	Pro/Ala/-	19/34/-	50.7
15	Ala/Thr/-	20/35/-	18.9
16	Asp/-/-	21/-/-	78.8
17	Glu/-/-	22/-/-	39.0
18	Glu/-/-	23/-/-	68.0
19	Met/-/-	24/-/-	71.3
20	Leu/-/-	25/-/-	3.7
21	Phe/-/-	26/-/-	42.0
22	Ile/-/-	27/-/-	0
23	Tyr/-/-	28/-/-	0
24	Ser/-/-	29/-/-	16.3
25	His/-/-	30/-/-	0
26	Tyr/-/-	31/-/-	0
27	Lys/-/-	32/-/-	0
28	Gln/-/-	33/-/-	0
29	Ala/-/-	34/-/-	38.7
30	Thr/-/-	35/-/-	0
31	Val/-/-	36/-/-	0
32	Gly/-/-	37/-/-	0

technical assistance in purifying ACBP and Bente Petersen for typing the manuscript.

REFERENCES

- Jeng, S. J. & Guillory, R. J. (1975) *J. Supramol. Struct.* **3**, 448–468
- Mikkelsen, J. & Knudsen, J. (1987) *Biochem. J.* **248**, 709–714
- Mikkelsen, J., Højrup, P., Nielsen, P. F., Roepstorff, P. & Knudsen, J. (1987) *Biochem. J.* **245**, 857–861
- Mogensen, I. B., Schulenberg, H., Hansen, H. O., Spener, F. & Knudsen, J. (1987) *Biochem. J.* **241**, 189–192
- Moonen, P., Haagsman, H. P., van Deenen, L. L. M. & Wirtz, K. W. R. (1979) *Eur. J. Biochem.* **99**, 439–445
- Nielsen, P. F., Klarskov, K., Højrup, P. & Roepstorff, P. (1988) *Biomed. Environ. Mass Spectrom.* **17**, 355–362
- Rasmussen, J. T., Børchers, T. & Knudsen, J. (1990) *Biochem. J.* **265**, 849–855
- Sanchez, M., Nicolls, D. G. & Brindley, D. N. (1973) *Biochem. J.* **170**, 565–574

Received 12 March 1990/29 May 1990; accepted 11 June 1990