

Fluorescently labelled bovine acyl-CoA-binding protein acting as an acyl-CoA sensor: interaction with CoA and acyl-CoA esters and its use in measuring free acyl-CoA esters and non-esterified fatty acids

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Long-chain acyl-CoA esters are key metabolites in lipid synthesis and β -oxidation but, at the same time, are important regulators of intermediate metabolism, insulin secretion, vesicular trafficking and gene expression. Key tools in studying the regulatory functions of acyl-CoA esters are reliable methods for the determination of free acyl-CoA concentrations. No such method is presently available. In the present study, we describe the synthesis of two acyl-CoA sensors for measuring free acyl-CoA concentrations using acyl-CoA-binding protein as a scaffold. Met²⁴ and Ala⁵³ of bovine acyl-CoA-binding protein were replaced by cysteine residues, which were covalently modified with 6-bromoacetyl-2-dimethylaminonaphthalene to make the two fluorescent acyl-CoA indicators (FACIs) FACI-24 and FACI-53. FACI-24 and FACI-53 showed fluorescence emission maximum at 510 and 525 nm respectively, in the absence of ligand (excitation

387 nm). Titration of FACI-24 and FACI-53 with hexadecanoyl-CoA and dodecanoyl-CoA increased the fluorescence yield 5.5- and 4.7-fold at 460 and 495 nm respectively. FACI-24 exhibited a high, and similar increase in, fluorescence yield at 460 nm upon binding of C₁₄–C₂₀ saturated and unsaturated acyl-CoA esters. Both indicators bind long-chain (> C₁₄) acyl-CoA esters with high specificity and affinity ($K_d = 0.6$ – 1.7 nM). FACI-53 showed a high fluorescence yield for C₈–C₁₂ acyl chains. It is shown that FACI-24 acts as a sensitive acyl-CoA sensor for measuring the concentration of free acyl-CoA, acyl-CoA synthetase activity and the concentrations of free fatty acids after conversion of the fatty acid into their respective acyl-CoA esters.

Key words: diagnostics, dissociation constant, intestinal fatty acid-binding protein.

INTRODUCTION

Non-esterified fatty acids (NEFAs) with acyl chains > 16 carbons are quantitatively the most important physiological energy source. By supplying 60–70% of cardiac energy requirement, circulating fatty acids represent the major energy source for cardiac tissue. The concentration of NEFAs in circulating blood is the rate determining factor in the regulation of fatty acid uptake [1] and has been shown to affect intracellular acyl-CoA concentrations [2]. Conversion of fatty acids into their CoA thioester derivatives is an obligatory step in the utilization of fatty acids for β -oxidation, complex lipid synthesis and post-translational protein modification. However, besides playing a key role in lipid metabolism, acyl-CoA esters have also been shown to act as regulators of enzyme activities, vesicular trafficking, hormone signalling, Ca²⁺ fluxes, ion channels and gene transcription (see [3] for review).

By influencing the circulating fatty acid levels and composition, dietary fatty acids specifically modulate the onset of various diseases, including cancer [4–6], atherogenesis [7] and coronary heart disease [8]. A still growing number of scientific reports indicate that accumulation of both intramuscular triacylglycerol and long-chain acyl-CoA (LCA) esters strongly correlate with the development of insulin resistance [9,10]. Defects in insulin

function caused by tissue-specific expression of lipoprotein lipase [11] and high fat feeding in rat are closely associated with accumulation of LCA [12]. A strong inverse correlation between glucose uptake and LCA content is observed in red quadriceps [13,14], and LCA content in human skeletal muscle correlates well with the measurement of whole body insulin action [12]. These observations have led to the suggestion that an initial increase in the content of LCA might be the primary event leading to insulin resistance. Increased levels of fatty acids and LCA have also been suggested to play an important role in both regulating insulin secretion and impaired β -cell function in late stages of type II diabetes [15,16].

LCA esters are highly amphiphatic molecules which bind non-specifically to proteins, test tube walls and partition into lipid membranes ($K = 1.5 \times 10^3$) [17]. The concentration of unbound acyl-CoA esters, which are presumed to be the regulatory species, is therefore likely to be different from the total concentration. Determination of free LCA concentrations *in vivo* and *in vitro* therefore have important applications in a wide variety of biochemical, biophysical, cell biological and physiological investigations. A number of HPLC, GLC and enzymic methods for determination of total acyl-CoA levels in tissue extracts and body fluids have been developed (see [18] for review). NEFA concentrations can conveniently be determined using the acrylo-

Abbreviations used: ACBP, acyl-CoA-binding protein; ADIFAB, acrylodated intestinal fatty acid-binding protein; DTT, dithiothreitol; FABP, fatty acid-binding proteins; FACI, fluorescence acyl-CoA indicator; LCA, long-chain acyl-CoA; NEFA, non-esterified fatty acid; TCA, trichloroacetic acid.

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dated intestinal fatty acid-binding protein (ADIFAB) [19]. However, a similar method is not currently available for determining free acyl-CoA levels. In the present study, we describe the construction and synthesis of a fluorescent acyl-CoA-binding protein (ACBP), which is capable of determining free acyl-CoA concentrations, monitoring the activity of acyl-CoA-producing enzymes and acts as a convenient sensor for the determination of NEFAs, provided that they are converted into acyl-CoA esters by an acyl-CoA synthetase.

MATERIALS AND METHODS

Reagents

Badan (6-bromoacetyl-2-dimethylaminonaphthalene) was obtained from Molecular Probes (Eugene, OR, U.S.A.). CoA was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). The plasmid pN3576 carrying the gene encoding the *Escherichia coli* acyl-CoA synthetase FadD was kindly provided by Dr Paul Black, Albany Medical College, Albany, NY, U.S.A. FACI-24 was obtained from Biosensor ApS (Årsløv, Denmark). All other reagents used were of analytical grade.

Site-directed mutagenesis and protein expression and purification

Site-directed mutagenesis of recombinant bovine ACBP was performed as described previously [20]. Briefly, adjacent 5'-phosphorylated oligonucleotides were designed on opposite DNA strands with the mutation encoded at the 5'-end of the upstream primer. PCR mutagenesis was performed by using the bovine ACBP open reading frame in pET3a as a template (10–50 ng) in a mixture consisting of 1.25 units of *Pfu* turbo polymerase (Stratagene, La Jolla, CA, U.S.A.), 50 pmol of each oligonucleotide and 200 μ M of each dNTP in *Pfu* reaction buffer [20 mM Tris/HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100 and 10 μ g/ml BSA]. The primers used for amplification of individual mutants are shown in Table 1. Prior to amplification, the template was denatured at 94 °C for 5 min, followed by 16 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 13 min. Controls did not receive any polymerase. Subsequently, the amplicon was extracted in phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and precipitated with ethanol. DNA was resuspended in 20 μ l of T4 ligase buffer (New England Biolabs, Frankfurt, Germany) containing T4 ligase (10 units) and incubated for 18 h at 16 °C. The samples were diluted to 50 μ l in *DpnI* digestion buffer [50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate and

1 mM dithiothreitol (DTT) (pH 7.9)] and incubated with *DpnI* (60 units) for 2 h at 37 °C. DNA (2–5 μ l) was then transformed into CaCl₂-competent DH5 α cells. Plasmid DNA was isolated by standard methods and plasmids carrying the correct change were identified by restriction analysis and DNA sequencing.

For large-scale production of recombinant protein, the bacteria were grown in a 4 litre fermenter as described previously [21]. The cells were harvested by centrifugation and frozen at –80 °C. Cells were thawed, resuspended in 0.9% NaCl/1 M acetic acid, sonicated and cleared by centrifugation for 20 min at 10000 g at 4 °C. The pH was adjusted to 7.0 with 1 M NaOH and the precipitated proteins were removed by centrifugation as described above. The cleared supernatant (approx. 140–160 ml) was divided in two and was loaded on to a Sephadex-G50 column (5 cm \times 80 cm; Amersham Biosciences, Copenhagen, Denmark), equilibrated and run with 10 mM Tris/HCl (pH 7.2) at 72 ml/h. The eluate was collected in 12 ml fractions. The fractions containing the ACBP peak were pooled, diluted to 5% (w/v) with freshly prepared 50% (w/v) trichloroacetic acid (TCA) and centrifuged as described above. The tube walls and the protein pellet were washed carefully with 10 mM TCA. The residual TCA was removed carefully and the protein pellet was dissolved in 30 mM Tris base/100 mM DTT and the pH adjusted to 7.2 with 30 mM Tris base. The solution was cleared by centrifugation and stored at –80 °C. Immediately before badan labelling, the frozen supernatant was thawed and loaded on to a Q-Sepharose HP column (1.5 cm \times 12 cm; Amersham Biosciences), pre-equilibrated with buffer A [10 mM Tris/HCl (pH 7.2)], and bound proteins were eluted with a linear gradient from 0–100% buffer B [400 mM NaCl in 10 mM Tris/HCl (pH 7.2); equivalent to a gradient of 0–400 mM NaCl], with a flow of 3 ml/min. The fractions containing ACBP were pooled, adjusted to a concentration of 100 mM with 1 M Tris/HCl (pH 7.2) and used directly for synthesis of badan-derivatized ACBP.

Badan labelling

The bovine recombinant Met²⁴Cys-, Met⁴⁶Cys-, Phe⁴⁹Cys- and Ala⁵³Cys-ACBPs were labelled with badan. Synthesis and handling of badan-derivatized proteins was carried out under dim light. To carry out the reaction, 1.1-fold molar excess of badan to ACBP was added over 10 min by continuous infusion from a 20 mM stock solution of badan (dissolved in dimethylformamide) to a 5 mg/ml solution of the individual proteins. The progress of the reaction was monitored by HPLC by injection of aliquots on to a Jupiter C₁₈ reverse-phase column [5 μ m particle size, 300 Å pore size (1 Å = 0.1 nm), 4.6 mm \times 250 mm; Phenomenex, Aschaffenburg, Germany] pre-equilibrated with buffer C (20% acetonitrile in water/0.05% TFA). Elution was carried out using a gradient from 15–100% buffer D (80% acetonitrile in water/0.05% TFA; equivalent to 20–80% acetonitrile) over a period of 15 min. Badan infusion was continued until all non-derivatized ACBP had disappeared. The reaction was then stopped by the addition of 1 mM DTT, and the reaction mixture was desalted into water on a Sephadex-G25 column (5 cm \times 25 cm) and freeze-dried. The location of the badan-derivatized amino acid was confirmed by tryptic digestion and separation of the tryptic peptides by reverse-phase HPLC using a water/acetonitrile/TFA solvent system, followed by mass determination and sequencing of the fluorescently labelled peptide. The resulting fluorescent acyl-CoA indicators (FACIs) were named FACI-24 (Met²⁴Cys-ACBP–badan), FACI-46 (Met⁴⁶Cys-ACBP–badan), FACI-49 (Phe⁴⁹Cys-ACBP–badan) and FACI-53 (Ala⁵³Cys-ACBP–badan) respectively, to signify the modified amino acid residue.

Table 1 Primers used for site-directed mutagenesis of bovine ACBP

Underlined bases represent the location of the mutated bases.

Mutation	Sequence
Met ²⁴ Cys	
Upstream	5'- <u>TGCTT</u> GTTTCATCTACTCTCACTACAAG
Downstream	5'-TTCTTCGTGGCCGGCTTGGTCTTC
Met ⁴⁶ Cys	
Upstream	5'- <u>TGCTT</u> GGAAGGTAAGGCTAAGGCTAAG
Downstream	5'-CCC GGCTCTTCGGTGTGATGTC
Phe ⁴⁹ Cys	
Upstream	5'- <u>TGCAAGG</u> GTAAGGCTAAGTGGGACGCT
Downstream	5'-GTCCAACATCCCGGGTCTTTCGGT
Ala ⁵³ Cys	
Upstream	5'- <u>TGCAAGT</u> GGGACGCTTGAACGAATTG
Downstream	5'-CTTACCCTTGAAGTCCAACATCCC

Acyl-CoA titration of FACI

Titration of FACIs with acyl-CoAs was performed using a SPEX FLOUROLOG (SPEX Industries Inc, Edison, NJ, U.S.A.) with excitation at 387 nm and emission at 460 nm, and both excitation and emission slits set to 4.5 nm. FACI (0.5–4.5 μM , as indicated) was dissolved in 1.5 ml of binding buffer [10 mM Hepes, 150 mM NaCl, 5 mM KCl and 1 mM Na_2HPO_4 (pH 7.4)] and titrated with 10 μM acyl-CoA dissolved in binding buffer, with or without FACI added, as indicated. Fluorescence emission values (counts per second) measured without FACI added to the ligand stock solution were corrected for titrant dilution.

Equilibrium binding analysis

The development of a mathematical equation describing the relationship between acyl-CoA concentration and FACI-24 fluorescence was performed essentially as described previously [19]. However, where ligand binding to ADIFAB induces both a fluorescence intensity increase at approx. 505 nm and a fluorescence intensity decrease at approx. 432 nm, ligand binding to FACI-24 only causes an increase in fluorescence at approx. 460 nm. Hence, the equation describing the relationship between the free concentration of acyl-CoA and the fluorescence is:

$$[\text{acyl-CoA}] = K_d \frac{F - F_{\min}}{F_{\max} - F} \quad (1)$$

where F is the fluorescence intensity at 460 nm and

$$[\text{acyl-CoA}_{\text{bound}}] = [\text{FACI}] \frac{F - F_{\min}}{F_{\max} - F_{\min}} \quad (2)$$

The derivation of an expression for the fluorescence value as a function of the acyl-CoA concentration was performed using the Scatchard equation as a starting point:

$$\frac{1}{[\text{acyl-CoA}_{\text{bound}}]} = \frac{K_d}{[\text{FACI}]} + \frac{1}{[\text{acyl-CoA}]} \quad (3)$$

Since $[\text{acyl-CoA}_{\text{free}}] = [\text{acyl-CoA}_{\text{total}}] - [\text{acyl-CoA}_{\text{bound}}]$ and recalling eqn 2:

$$\frac{1}{[\text{acyl-CoA}_{\text{bound}}]} = \frac{K_d}{[\text{FACI}]} + \frac{1}{[\text{acyl-CoA}_{\text{total}}] - [\text{acyl-CoA}_{\text{bound}}]} + \frac{1}{[\text{FACI}]}$$

$$\Downarrow$$

$$\frac{1}{[\text{FACI}] \frac{F - F_{\min}}{F_{\max} - F_{\min}}} = \frac{K_d}{[\text{FACI}]} + \frac{1}{[\text{acyl-CoA}_{\text{total}}] - [\text{FACI}] \frac{F - F_{\min}}{F_{\max} - F_{\min}}} + \frac{1}{[\text{FACI}]}$$

This equation can be solved for F , yielding

$$F = \frac{(F_{\max} - F_{\min}) \sqrt{K_d^2 + (2[\text{acyl-CoA}] + 2[\text{FACI}]K_d + [\text{acyl-CoA}]^2 - 2[\text{FACI}][\text{acyl-CoA}] + [\text{FACI}]^2) + (F_{\max} - F_{\min})K_d + (-[\text{acyl-CoA}] - [\text{FACI}])F_{\max} + ([\text{acyl-CoA}] - [\text{FACI}])F_{\min}}}{2[\text{FACI}]} \quad (4)$$

This equation has been used to fit the titration data.

Titration data analysis

Fluorescence emission intensity is presented as a function of the acyl-CoA:FACI-24 ratio, and fitted to the mathematical model (described by eqn 4) using DataFit® 7.1 software (Oakdale

Engineering, Oakdale, PA, U.S.A.). Four parameters were fitted, namely the point of acyl-CoA/FACI equimolarity (B), initially set to 1, the maximum fluorescence emission intensity (F_{\max}), the minimum fluorescence emission intensity (F_{\min}) and the K_d value for the acyl-CoA. For each acyl-CoA, at least two titrations and fittings were performed. To compare two or more titrations of the same acyl-CoA together and obtain a K_d value, both axes of the raw data were normalized using the fitted values of B , F_{\max} and F_{\min} in this way: X axis standard = (raw X axis values)/ B fitted; and Y axis standard = [(raw Y axis values) - F_{\min} fitted]/[F_{\max} fitted - F_{\min} fitted].

The normalized data were fitted using DataFit®. For short-chain (< $\text{C}_{8:0}$ -CoA) acyl-CoA esters, titration curves were found to lack a significant point of saturation, causing the fitting procedure to yield very high B values (2–3). This could be overcome by removing B from the list of parameters to be fitted, and instead setting it to a constant value of 1 and fitting again. This was done for both the raw and the standardized data.

Determination of acyl-CoA synthetase activity and quantification of NEFA concentration

Determination of acyl-CoA synthetase activities and the concentration of free NEFAs were performed in black 96 multi-well plates using a Wallac Victor² Multilabel Reader (Wallac Oy, Turku, Finland). The reaction mixture (200 μl) contained 3 μM FACI-24 in 200 mM Tris/HCl (pH 7.4), 1 mM DTT, 2 mM EDTA, 4 mM MgCl_2 , 4 mM ATP, 60 μM CoA, 0.03 unit/ml acyl-CoA synthetase (GST-FadD) and 3 μM BSA. NEFA standard solutions were made as follows: fatty acids (1.26 μmol), dissolved in 1 ml of ethanol, were neutralized with equimolar amounts of 0.1 M NaOH and diluted with 4 ml of 96% ethanol to give a final concentration of 252 μM . The stock solution was diluted with 96% ethanol and added in 5 μl portions to the final reaction mixture in the multi-well microtitre plates. The plates were covered with parafilm and aluminium foil and allowed to incubate on an orbital shaker for 2 h at 20 °C. Fluorescence emission was measured at 460 nm (excitation at 390 nm) using a Wallac Victor² Multilabel Reader.

Production of GST-FadD

Recombinant *E. coli* fatty acyl-CoA synthetase FadD was expressed as an N-terminal GST fusion protein. The open reading frame of FadD was amplified using the pN3576 plasmid as a template [19] and specific oligonucleotides 5'-CACGGATCCATGAAGAAGGTTTGGCTTAACC-3' and 5'-CACGAATTCTCAGGCTTTATTGTCCACTTTG-3', carrying either a *Bam*HI or *Eco*RI restriction site (underlined) respectively. The Expand High Fidelity PCR System was used as described by the manufacturer (Roche, Hvidovre, Denmark). The PCR product

was digested with *Eco*RI and *Bam*HI and ligated into the pGEX-2TK vector (Amersham Biosciences) using standard techniques. The recombinant GST fusion protein was expressed in the *E. coli* BL21(DE3)p*Lys*S strain and purified essentially as described by the manufacturer (Amersham Biosciences), except that CoA (10 mM) was included in all buffers including the elution buffer.

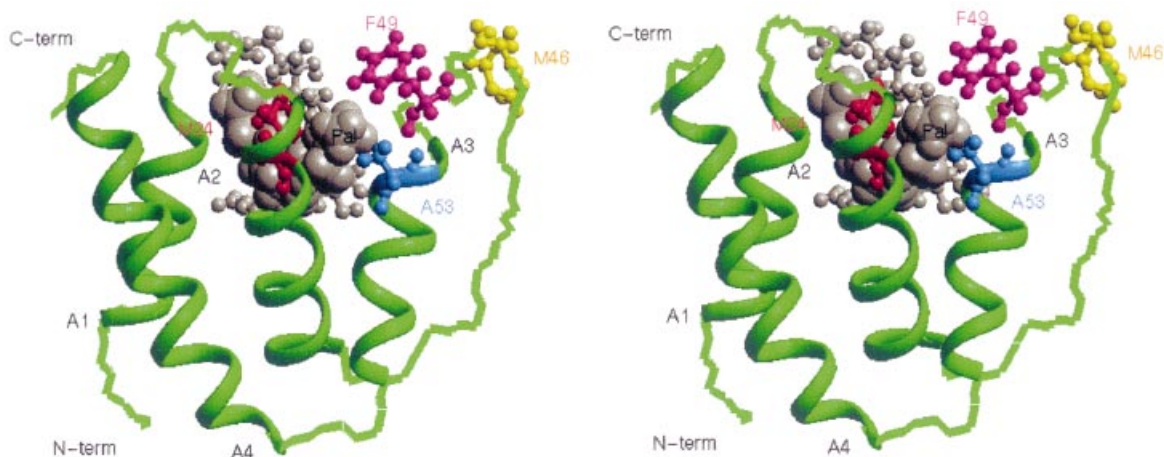


Figure 1 Bovine ACBP/C_{16:0}-CoA tertiary structure

ACBP is shown as backbone, the CoA head group (grey, ball-and-stick) and the acyl chain (grey, spacefill). The modified residues, Met²⁴ (M24; red), Met⁴⁶ (M46; yellow), Phe⁴⁹ (F49; magenta) and Ala⁵³ (A53; blue) are highlighted with ball-and-stick models. The positions of α -helices (A1, A2, A3 and A4) are also indicated. C-term, C-terminus; N-term, N-terminus.

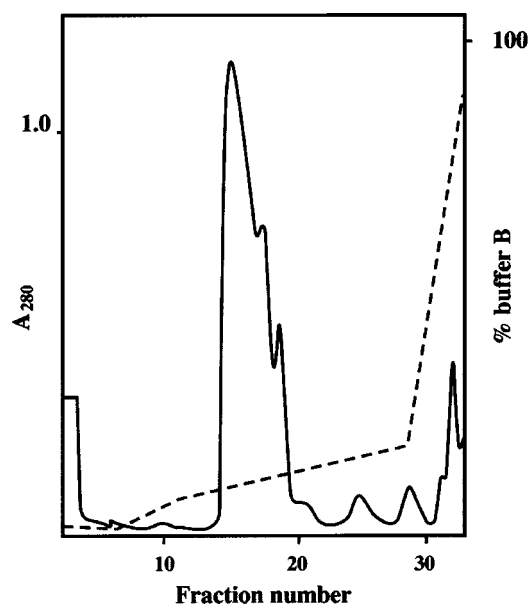


Figure 2 Q-Sepharose HP ion-exchange separation of bovine Met²⁴Cys-ACBP derivatives

The TCA-precipitated protein was dissolved in Tris-base and pH adjusted to 7.2 and loaded on to a Q-Sepharose HP column (1.5 cm \times 12 cm) pre-equilibrated with buffer A. Proteins were eluted with a gradient of 0–100% buffer B at a flow rate of 3 ml/min. See Materials and methods section for further details. Solid line, A_{280} ; dashed line, percentage of buffer B.

RESULTS

Protein expression and modification

Since fatty acid-binding proteins (FABPs) binds both fatty acids and acyl-CoA esters, the fluorescently labelled FABP ADIFAB cannot be used to measure the formation of acyl-CoA esters from NEFAs, because both ligands will generate a fluorescence emission upon binding. The advantage of using ACBP as a scaffold for making an acyl-CoA sensor is its very high specificity and affinity for acyl-CoA esters. Native bovine ACBP does not

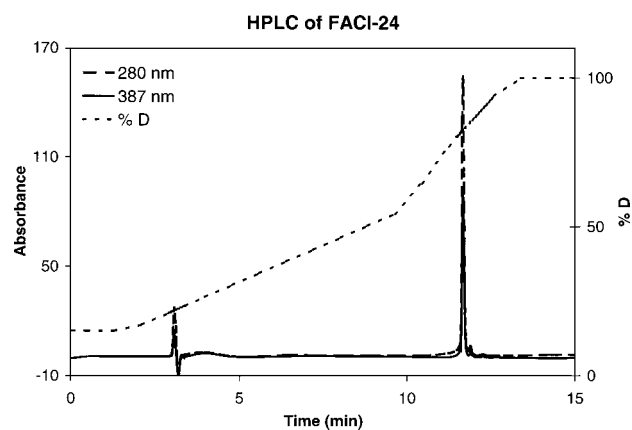


Figure 3 Analysis of FACI-24 by reverse-phase HPLC

The desalted reaction product from synthesizing FACI-24 was analysed on a Jupiter C₁₈ reverse-phase column [5 μ m particle size, 300 \AA pore size (1 \AA = 0.1 nm), 4.6 mm \times 250 mm] pre-equilibrated with buffer C and eluted with a gradient from 15–100% buffer D as shown. The results show that the product only contains one species and that the A_{280} (dashed line) and A_{387} (solid line) absorbing materials co-elute.

contain any cysteine residues. The strategy behind the design of an acyl-CoA sensor was to replace selected amino acid residues with cysteine residues and subsequently modify this residue using the thiol-reactive fluorescent probe badan. Met²⁴ and Ala⁵³, which interact with carbons 12–16 of hexadecanoyl-CoA, and Phe⁴⁹, positioned at the bottom of the binding pocket interacting with the Ω -methyl group of this ligand [22], were chosen for modification (Figure 1). Positioning of an environmentally sensitive group in one of these positions would therefore be expected to create ligand-sensitive probes. Met⁴⁶, which is located outside the hydrophobic binding pocket in the loop between helix 2 and 3, was chosen as a negative control, which should not be sensitive to ligand binding. Expression of the cysteine-modified bovine ACBPs in *E. coli* DH5 α with the gene inserted in the pKK233-3 expression vector, as described previously for native

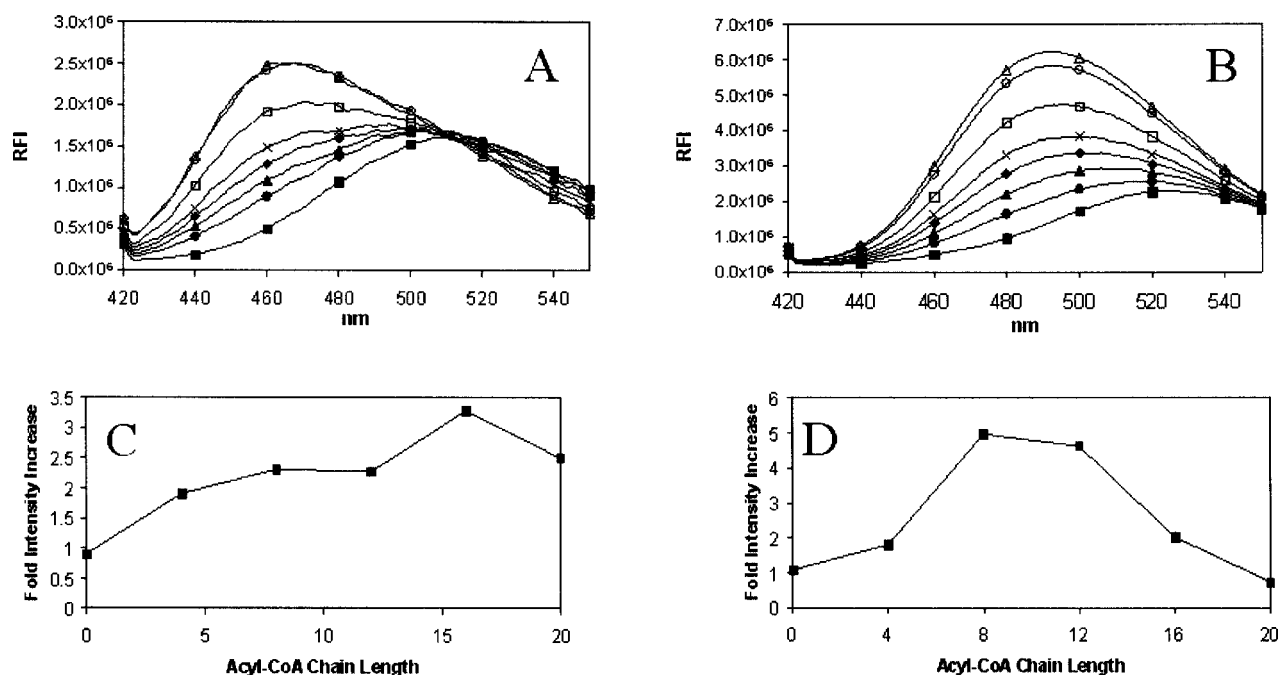


Figure 4 Fluorescence emission spectra (A and B) and fluorescence emission intensity dependence of acyl-CoA chain length (C and D) for FACI-24 (A and C) and FACI-53 (B and D)

Fluorescence emission spectra from titration of FACI-24 with C_{16} -CoA (A) and FACI-53 with C_{12} -CoA (B). Ligand concentrations were 0 (■), 0.3 (●), 0.6 (▲), 0.9 (◆), 1.2 (×), 1.8 (□), 2.7 (○) and 3.3 (△) μ M hexadecanoyl-CoA and dodecanoyl-CoA respectively. Acyl-CoA chain-length dependence of fluorescence emission maximum for FACI-24 (C) and FACI-53 (D) at saturating ligand concentrations. Excitation was at 387 nm and emission at 460 nm. For experimental details see Materials and methods section.

ACBP, was unsuccessful (J. Knudsen, unpublished work). The bacteria grew very slowly or expressed only low levels of the cysteine-modified ACBP proteins, indicating that they were toxic to DH5 α . Expression of the cysteine-containing proteins from the pET3a vector in the *E. coli* strain BL21(DE3)pLysS resulted in a high yield of recombinant proteins. However, following purification we found that the introduced cysteine residue was partially modified, a phenomenon which was most pronounced with Ala⁵³Cys-ACBP. Approx. 50% of the Ala⁵³Cys-ACBP produced was esterified with CoA, as shown by MS (result not shown). The expressed Met²⁴Cys-ACBP contained two modified versions that could be separated from the unmodified form by Q-Sepharose ion-exchange chromatography (Figure 2). The final unmodified product was shown to have the correct molecular mass as determined by MS (9883 Da; results not shown). Wild-type recombinant bovine ACBP did not react with badan under the reaction conditions used (results not shown); however, modification of the cysteine residue in Met²⁴Cys-ACBP caused a second group to react with badan. To prevent modification of this group, a 1.1-fold molar excess of badan to Met²⁴Cys-ACBP was infused into the reaction mixture at a controlled rate. Using this procedure, the Met²⁴Cys-ACBP was found to be completely modified to give only one product (Figure 3). The small amount of double-derivatized protein was insoluble and was completely absorbed during desalting. MS showed that the final product only contained one protein species with a molecular mass of 10095 Da, confirming that Met²⁴Cys-ACBP had only been modified with one badan. Tryptic digestion of FACI-24 resulted in two badan-derivatized peaks with molecular masses of 2155.71 and 1865.26 Da respectively. The unmodified tryptic peptide Thr¹⁷-Lys³² of Met²⁴Cys-ACBP has a predicted molecular mass of 1943.19 Da. The molecular masses of the two peaks, corrected

for the badan group (211.09 Da), are 1944.62 and 1654.17 Da respectively. The 2155.71 Da peptide therefore corresponds to the Thr¹⁸-Lys³² peptide, and the 1865.26 Da peptide is the same peptide which has lost the C-terminal Thr³¹ and Lys³². These data confirm that FACI-24 is derivatized with one badan at position Cys²⁴. The Ala⁵³Cys-ACBP variant eluted as two completely separate peaks from the ion-exchange column. MS showed that the second peak was 767 mass units larger, corresponding to the molecular mass of CoA. As expected, this extra mass could be removed by incubation with 100 mM DTT. This indicates that 30–40% of Cys⁵³ in Ala⁵³Cys-ACBP was covalently modified with CoA, through a disulphide bridge, by *E. coli*. Met⁴⁶Cys- and Phe⁴⁹Cys-ACBP were both determined to have the correct molecular mass. Their badan derivatives FACI-46, FACI-49 and FACI-53 was synthesized and characterized as described for FACI-24 above.

Ligand binding

Fluorescence titration emission spectra from 415–550 nm (excitation at 390 nm) were performed for CoA, $C_{4:0}$ -, $C_{8:0}$ -, $C_{12:0}$ -, $C_{16:0}$ - and $C_{20:0}$ -CoA with all four mutated and badan-modified proteins. FACI-46 and FACI-49 did not show any emission spectra changes with any of the ligands (results not shown) and were therefore not investigated further.

Titration of FACI-53 dissolved in binding buffer with the above-mentioned acyl-CoA esters produced a smooth downward shift in fluorescence emission maximum from 525 nm when no ligand was present to 508, 503, 490, 492, 496 and 514 with increasing concentrations of CoA, $C_{4:0}$ -, $C_{8:0}$ -, $C_{12:0}$ -, $C_{16:0}$ - and $C_{20:0}$ -CoA respectively (results not shown). Titration with $C_{16:0}$ -CoA is shown in Figure 4(B). The magnitudes of emission at

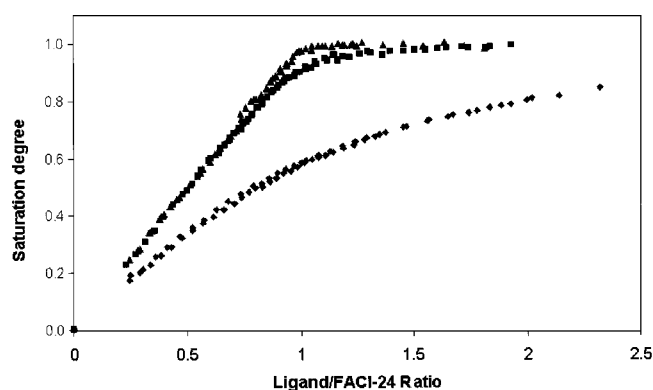


Figure 5 Normalized fitted binding curves for titration of FACI-24 with $C_{8:0}$ -, $C_{12:0}$ - and $C_{16:0}$ -CoA

FACI-24 [$1 \mu\text{M}$ in 10 mM HEPES/150 mM NaCl, 5 mM KCl/1 mM NaH_2PO_4 (pH 7.4)] was titrated with increasing amounts of $C_{8:0}$ -CoA (\blacklozenge), $C_{12:0}$ -CoA (\blacksquare) or $C_{16:0}$ -CoA (\blacktriangle) in the above buffer. Fluorescence values were adjusted for titrant dilution and mathematically fitted (see Materials and Methods section). Saturation degree (ratio of acyl CoA–FACI-24 complex to total FACI-24) was calculated from eqn 2. Results show an overlay of two independent titrations with each ligand.

Table 2 Dissociation constants for binding of CoA and acyl-CoA esters to FACI-24

FACI-24 ($1.5 \mu\text{M}$) was titrated with increasing CoA and acyl-CoA concentrations as shown in Figure 5. The data were fitted and K_d calculated as described in the Materials and method section. Results are means \pm S.E.M.

Ligand	K_d (nM)
CoA	2448.70 ± 248.60
$C_{4:0}$ -CoA	1496.80 ± 222.60
$C_{8:0}$ -CoA	342.00 ± 31.90
$C_{10:0}$ -CoA	61.80 ± 2.40
$C_{12:0}$ -CoA	10.20 ± 0.81
$C_{14:0}$ -CoA	1.66 ± 0.26
$C_{16:0}$ -CoA	0.65 ± 0.26
$C_{18:0}$ -CoA	1.65 ± 0.83
$C_{18:1}$ -CoA	0.59 ± 0.19

495 nm were increased 1.1-, 1.8-, 4.8-, 4.7-, 2.2- and 0.8-fold (Figure 4D), in the same order, demonstrating that FACI-53 gives the strongest signal for $C_{8:0}$ - to $C_{12:0}$ -acyl chains.

The emission maximum of FACI-24 without ligand added was 510 nm and titration with CoA, $C_{4:0}$ -, $C_{8:0}$ -, $C_{12:0}$ -, $C_{16:0}$ - and $C_{20:0}$ -CoA resulted in a 0, 16, 34, 42, 46 and 46 nm downshift in emission maximum respectively (results not shown). $C_{16:0}$ -CoA induced a 5.5-fold increase in emission at 464 nm (Figure 4A).

Titration of FACI-24 with increasing concentrations of $C_{8:0}$ -, $C_{12:0}$ - and $C_{16:0}$ -CoA resulted in a gradual increase in emission fluorescence at 460 nm (Figure 5), providing a sensitive measure for acyl-CoA binding. The curves represent the best-fit analysis from two independent experiments calculated and normalized as described above. The dissociation constants (K_d) for binding of the individual ligand to FACI-24 calculated from the binding curves are listed in Table 2. The FACI-24-binding affinity increased dramatically when the length of the acyl chain increased from C_8 to C_{10} and C_{12} , whereas a smaller, but significant, drop in K_d is seen by increasing chain length from C_{12} to C_{14} . C_{14} - to C_{18} -saturated acyl-CoA esters and $C_{18:1}$ -CoA binds with similar and very high affinities to FACI-24. FACI-24 binds acyl-CoAs

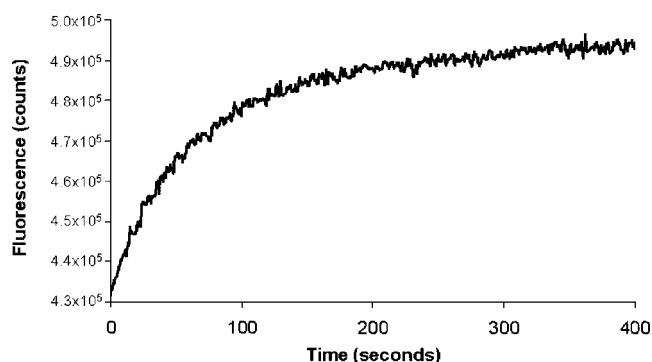


Figure 6 Determination of GST–FadD activity using FACI-24 as a sensor for the rate of acyl-CoA formation

The reaction mixture (1.5 ml) contained $3 \mu\text{M}$ FACI-24 in 200 mM Tris/HCl (pH 7.4), 1 mM DTT, 2 mM EDTA, 4 mM MgCl_2 , 4 mM ATP, $60 \mu\text{M}$ CoA, 0.03 unit/ml acyl-CoA synthetase (GST–FadD), $3 \mu\text{M}$ BSA and $100 \mu\text{M}$ sodium palmitate. The reaction was followed by monitoring the increase in emission at 460 nm (excitation 387 nm) over time.

with similar affinity and specificity as native unmodified bovine ACBP. The K_d for binding of $C_{12:0}$ - and $C_{16:0}$ -CoA to native ACBP, as determined by isothermal titration microcalorimetry in 25 mM ammonium acetate (pH 6.0), is 5.0 and 2.0 nM respectively [23,24]. FACI-24 is highly specific for binding LCA esters; however, NEFAs do not bind and do not affect fluorescence emission at any wavelength (results not shown). FACI-24 binds free CoA with a similar low affinity ($K_d = 2.5 \mu\text{M}$) as native bovine ACBP ($K_d = 2 \mu\text{M}$; [25]). CoA induces a smaller increase (3-fold) in FACI-24 fluorescence emission at 470 nm than $C_{14:0}$ - to $C_{18:0}$ -CoA, which induce 5- to 6-fold increases in fluorescence. The relative emission change induced by binding CoA and C_2 - to $C_{18:0}$ -CoA to FACI-24 are shown in Figure 4(C).

The shift in the fluorescence emission maximum from 525 nm to 460 nm upon ligand binding indicates that the badan group is shifted from a more hydrophilic environment to a more hydrophobic environment [4]. The precise mechanism of interaction of the ligand with the badan group is unknown. However, the fact that the interaction of $C_{12:0}$ - to $C_{18:0}$ -CoA esters results in very similar increases in the emission yield at 460 nm independent of the length of the acyl chain, indicates that it is either the early part of the acyl chain or the CoA head group which interacts with the badan group. This interpretation is supported by folding studies which suggest that the pantetheine part of the CoA head group interacts with the acyl chain of hexadecanoyl-CoA bound to native bovine ACBP [4]. The final evidence for this interaction will have to await determination of the tertiary structure of apo and holo FACI-24, which is currently in progress.

The very high fluorescence yield and binding affinity for acyl-CoAs with a length of the chain $> C_{12:0}$ -CoA ($K_d = 1\text{--}2$ nM), combined with the low binding affinity ($K_d = 2 \mu\text{M}$) and low fluorescence yield with CoA, makes FACI-24 a potential sensor for quantification of mixtures of $C_{12:0}$ - to $C_{20:0}$ -CoA esters synthesized by acyl-CoA synthetase.

Measurement of acyl-CoA synthetase activity

The activity of the fatty acyl-CoA synthetase FadD was followed by measuring the increase in the fluorescence intensity at 460 nm in a cuvette with reaction mixture containing FACI-24 and GST–FadD (Figure 6). The results show that emission at 460 nm increased in a linear fashion over time. The addition of the

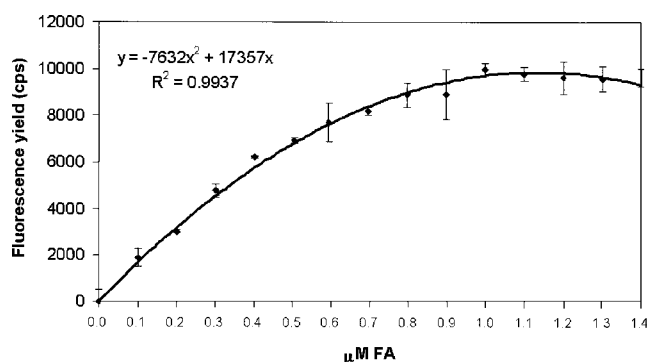


Figure 7 Fatty acid standard curve

Various concentrations of sodium palmitate (FA), dissolved in ethanol (5 μ l), were added to 200 μ l of reaction mixture [200 mM Tris/HCl (pH 7.4), 1 mM DTT, 2 mM EDTA, 4 mM MgCl₂, 4 mM ATP, 60 μ M CoA, 0.03 unit/ml acyl-CoA synthetase (GST–FadD) and 1 μ M BSA] containing 1 μ M FACI-24 and the fluorescence yield (in counts per second, cps) was determined as described in the Materials and methods section. Results are means \pm S.D. The equation of the fitted line is indicated along with the correlation coefficient (R^2).

reaction mixture increased background fluorescence; however, this did not affect the sensitivity of the sensor.

Incubating GST–FadD in reaction mixture containing FACI-24 (1.0 μ M) and increasing sodium palmitate concentrations (Figure 7) showed that FACI-24 produced an almost linear increase in emission at 460 nm in response to the increased concentration of NEFAs in the reaction mixture. This suggests that FACI-24 might be used for establishing a very simple and highly sensitive assay for determining NEFA concentrations in biological fluids and tissue extracts.

DISCUSSION

LCA esters are not only key metabolites in lipid synthesis and β -oxidation, but also important regulators of metabolism, insulin secretion, vesicular trafficking and gene expression [3]. A finely tuned balance between the activities of acyl-CoA synthetases and hydrolases control the free acyl-CoA concentrations. Specialized ACBPs are believed to channel acyl-CoA to their site of action. A key tool in the study of the regulatory functions of acyl-CoA esters is a reliable method for the determination of free acyl-CoA concentrations. However, no method is presently available for measuring free acyl-CoA concentrations. In the current study, we present the synthesis of two acyl-CoA sensors for measuring free acyl-CoA concentrations, using ACBP as a scaffold.

The present results demonstrate that bovine ACBP provides a useful scaffold for synthesizing acyl-CoA sensors with different specificity. The fluorescence emission yield of FACI-53 is highly specific for C_{8:0}- to C_{12:0}-CoAs. FACI-24 provides an all-round acyl-CoA sensor for measuring low concentrations of free acyl-CoAs with chain lengths longer than 14 carbon atoms. Determination of binding constants for high affinity ACBPs ($K_d < 10$ nM) is difficult by conventional techniques, such as isothermal titration calorimetry [3]. This problem can now be overcome with FACI-24 by using the approach adopted by Kleinfeld and co-workers [19] for determining binding constants for FABPs using fluorescently labelled ADIFAB. Using the same experimental approach, FACI-24 provides an excellent tool for determining binding affinities for ACBPs with K_d s in the low nanomolar range. We have shown previously that the K_d for binding of hexadecanoyl-CoA to bovine ACBP gradually increased from 2–1500 nM by successively mutating Lys³² to

arginine, alanine and glutamic acid respectively [22]. We are currently making FACI-24 carrying these changes to produce FACIs covering the binding affinity range from 0.5 nM–4 μ M. The results from measuring FadD activity show that FACI-24 is an excellent sensor for the determination of the activity of acyl-CoA-producing enzymes. FACI-24 binds C₁₄–C₁₈ saturated and unsaturated acyl-CoA esters with similar affinity and fluorescent emission yield at 460 nm. This suggests that FACI-24 might be used as a convenient and sensitive sensor for quantification of NEFAs in biological fluids, after conversion of the acids into acyl-CoA esters.

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