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The purification and properties of ox liver short-chain acyl-CoA dehydrogenase

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The FAD-containing short-chain acyl-CoA dehydrogenase was purified from ox liver mitochondria by using $(NH_4)_2SO_4$ fractionation, DEAE-Sephadex A-50 and chromatofocusing on PBE 94 resin. The enzyme is a tetramer, with a native M_r of approx. 162000 and a subunit M_r of 41000. Short-chain acyl-CoA dehydrogenases are usually isolated in a green form. The chromatofocusing step in the purification presented here partially resolved the enzyme into a green form and a yellow form. In the dye-mediated assay system, the enzyme exhibited optimal activity towards 50 μM-butyryl-CoA at pH 7.1. Kinetic parameters were also determined for a number of other straight-chain acyl-CoA substrates. The u.v.- and visible-absorption characteristics of the native forms of the enzyme are described, together with complexes formed by addition of butyryl-CoA, acetoacetyl-CoA and CoA persulphide.

The mammalian mitochondrial acyl-CoA dehydrogenases are a group of soluble FAD-linked enzymes that introduce a *trans*-$\alpha\beta$-double-bond into saturated acyl-CoA substrates during β-oxidation. Long-chain (palmitoyl), general (medium-chain) and short-chain (butyryl) acyl-CoA dehydrogenases were originally described in the 1950s (Seubert & Lynen, 1953; Green *et al.*, 1954; Crane *et al.*, 1956*b*; Hauge *et al.*, 1956).

Despite their obvious importance in mammalian metabolism, these enzymes have been somewhat neglected since their discovery, mainly because of the difficulty of separating such physically similar proteins. None of the original purification methods gave yields greater than 4%. Non-availability of substrates and other problems in enzymic assay have also contributed to this neglect.

With recent advances in protein purification techniques, interest in these enzymes has revived and new purifications have been reported for the general acyl-CoA dehydrogenase (GAD) (Hall & Kamin, 1975; Thorpe *et al.*, 1979). However, apart from a partial characterization of the monkey liver enzyme (Hoskins, 1966), the short-chain acyl-CoA dehydrogenase (SCAD) has continued to receive remarkably little attention.

Further studies on purified preparations of the acyl-CoA dehydrogenases are required for a more complete understanding of β-oxidation and their suggested role in the control of this pathway (Davidson & Schulz, 1982). The relationship between the straight-chain acyl-CoA dehydrogenases and the more-recently discovered branched-chain acyl-CoA dehydrogenases involved in amino acid degradation (Noda *et al.*, 1980) still has to be explored. Interesting questions with regard to the role of the flavin in catalysis are also raised by this group of related enzymes.

Acyl-CoA dehydrogenases bind their substrates very tightly. Steyn-Parvé & Beinert (1958*a*) showed that labelled substrate would remain associated with the enzyme after dialysis, $(NH_4)_2SO_4$ precipitation and several other separation procedures.

Upon addition of a reduced substrate, a mammalian acyl-CoA dehydrogenase typically forms a stable intermediate complex. The absorption spectrum shows partial reduction of the flavin (decreased A_{450}) and formation of a new long-wavelength band at 560nm. These absorbance changes were originally ascribed to the formation of a flavin–semiquinone (Beinert, 1957), but later work suggests that a flavin–substrate charge-transfer complex is formed (Massey & Ghisla, 1974; Hall *et al.*, 1979). The stability of the complex to O_2 contrasts with the situation for many other flavoproteins (notably the oxidases) whose substrate-reduced forms are immediately re-oxidized by molecular O_2. Stabilization against O_2 is important in reserving substrate-derived reducing equivalents specifically for electron-transferring flavo-

Abbreviations used: SCAD, short-chain acyl-CoA dehydrogenase (EC 1.3.99.2); GAD, general acyl-CoA dehydrogenase (EC 1.3.99.3).

protein, the physiological acceptor, whence they are passed into the electron-transport chain with the generation of ATP (Crane & Beinert, 1956).

Interestingly, the SCAD from the anaerobic bacterium *Megasphaera elsdenii* (Engel & Massey, 1971*a*) does not stabilize the substrate-reduced flavin against re-oxidation. We are thus in a position to assess the role of the charge-transfer complex in acyl-CoA dehydrogenase catalysis by the comparison of these two otherwise very similar enzymes.

In the present paper we describe a purification for a mammalian (ox liver) SCAD giving a good yield from manageable quantities of tissue, and characterize some of its basic properties.

Materials and methods

Synthesis of acyl-CoA compounds

Butyryl-CoA and propionyl-CoA were synthesized from acid anhydrides (Simon & Shemin, 1953; Stadtman, 1954). Acetoacetyl-CoA was synthesized from freshly distilled diketen (Michal & Bergmeyer, 1963). All other acyl-CoA compounds were synthesized by the method of Kawaguchi *et al.* (1981) from fatty acids with carbonyldi-imidazole.

All acyl-CoA compounds (except octanoyl-CoA and dodecanoyl-CoA) were purified on a column (1 cm × 15 cm) of DEAE-Sephadex A-25 (Williamson & Engel, 1984). Purified acyl-CoA compounds were stored freeze-dried or frozen in solution at −70°C. All saturated acyl-CoA compounds were assayed by their A_{260} ($\varepsilon = 15.4\,mM^{-1}\cdot cm^{-1}$) and by quantitative determination of released CoA with 5,5′-dithiobis-(2-nitrobenzoic acid) after alkaline hydrolysis. Acetoacetyl-CoA was assayed by the method of Stern (1956).

Reagents

Butyric anhydride, propionic anhydride, 5,5′-dithiobis-(2-nitrobenzoic acid), 2,6-dichlorophenol-indophenol, guanidinium chloride (AristaR), 2-mercaptoethanol and L-histidine hydrochloride were obtained from BDH Chemicals, Atherstone, Warwicks., U.K. Diketen and carbonyldi-imidazole were supplied by Aldrich Chemical Co., Gillingham, Dorset, U.K. All fatty acids, CoA, phenazine ethosulphate, phenylmethanesulphonyl fluoride and poly(ethylene glycol) (M_r 20000) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Coomassie Brilliant Blue was from Shandon Southern Products, Runcorn, Cheshire, U.K. $Na_2S_2O_4$ was obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Chromatographic materials

All chromatographic materials were pretreated, used and stored according to the manufacturer's directions. Sephadex G-150, DEAE-Sephadex A-25 and A-50, PBE 94 resin and Polybuffer 74 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel P-2 was purchased from Bio-Rad Laboratories, Watford, Herts., U.K.

Instrumentation

All absorbances, spectra and enzymic reaction rates were measured in the thermostatically controlled cuvette holder of a Varian Cary 219 spectrophotometer (Varian Associates, Walton-on-Thames, Surrey, U.K.).

Salt concentrations in column eluates were measured, with reference to a standard curve, with a conductivity meter (Electronic Switchgear, Hitchin, Herts., U.K.).

Slab gels, run on a Protean electrophoresis system (Bio-Rad Laboratories), were stained with Coomassie Brilliant Blue.

Protein assay

Protein concentrations during the purification were determined by the method of Bradford (1978), with reference to a bovine serum albumin standard curve.

Preparation of mitochondria

The method used in the present work is a modification of that of Crane *et al.* (1956*a*). All procedures were performed at 4°C with precooled solutions and centrifuge rotors. A 1 kg portion of liver from a freshly killed ox was transported on ice to the laboratory, and was minced and homogenized in 2.6 litres of 10 mM-potassium phosphate buffer, pH 7.6 containing 0.25 M-sucrose, for 45 s at top speed in a Waring Blendor. The homogenate was centrifuged at 1350*g* for 7 min (2000 rev./min, 6 × 1-litre rotor) in an MSE Mistral 61 centrifuge. To the supernatant, filtered through three layers of muslin, 600 ml of 0.9% KCl was added. The mitochondria were harvested at 13000*g* for 10 min in a Sorvall RC-5 centrifuge (9000 rev./min, GSA rotor). The soft pellets were gently blended into 1 litre of 0.9% KCl and centrifuged at 13000*g* for 30 min (9000 rev./min, GSA rotor). The mitochondria were either used immediately or stored at −20°C until required.

Extraction of soluble proteins from mitochondria

The mitochondria were blended into 3 vol. (3 ml/g wet wt.) of 25 mM-potassium phosphate buffer, pH 6.5, containing 0.3 mM-EDTA and 1 mM-phenylmethanesulphonyl fluoride and sonicated in 200 W bursts for 4 × 30 s (with 30 s cooling between bursts) in a salt/ice bath with a Branson or Heat Systems W-225R sonicator. The sonicated material was centrifuged at 65000*g* for 1 h (25000 rev./min, 10 × 100 ml rotor) in an MSE

Prepspin 50 centrifuge, to obtain a clear pink supernatant. For larger amounts of mitochondria it was found convenient to centrifuge at 27000*g* for 1½h (13000rev./min, GSA rotor). This gave a more-turbid supernatant, which was partially clarified by the first $(NH_4)_2SO_4$ precipitation.

Enzyme assay

The assay method used in the present work is adapted from that of Engel & Massey (1971*a*). For assays performed during the purification, 10μl of enzyme solution was pipetted into 1ml of 120mM-potassium phosphate buffer, pH7.1, containing 0.001% dichlorophenol-indophenol and 50μM-butyryl-CoA at 25°C. Any background decrease in absorbance at 600nm was measured ($\varepsilon = 21.0 mM^{-1} \cdot cm^{-1}$) (Armstrong, 1964). The full rate of enzymic dichlorophenol-indophenol reduction was initiated on addition of phenazine ethosulphate (Williamson & Engel, 1984) to a final, saturating, concentration of 0.6mM. For kinetic experiments the above procedure was used, with appropriate variations in buffer conditions and nature and concentration of acyl-CoA.

Determination of the absorption coefficient for enzyme-bound FAD

The tightly bound FAD was removed from the enzyme and quantified by two methods. In the first, a sample of enzyme was treated with 5M-guanidinium chloride. The absorption coefficient was calculated by comparing the original A_{450} of the enzyme with that of the released FAD, with appropriate compensation for the increase in FAD absorbance caused by the presence of guanidinium chloride (Thorpe *et al.*, 1979). The second method was to release the flavin from a sample of enzyme of known flavin absorbance with 5% (w/v) trichloroacetic acid. After centrifugation, the pellet was further extracted with 5% trichloroacetic acid. The extracts were pooled, extracted five times with diethyl ether and made up to a known volume, and the released flavin was quantified by its A_{450} (Mayhew & Massey, 1969).

Determination of the enzyme's M_r and subunit structure

The enzyme's native M_r was determined by using a column (1.5cm × 82cm) of Sephadex G-150 equilibrated in 50mM-potassium phosphate buffer, pH7.6, containing 0.3mM-EDTA and calibrated with the following proteins: horse cytochrome *c* (M_r 12400), *M. elsdenii* flavodoxin (M_r 15000), bovine pancreas chymotrypsin (M_r 25000), ovalbumin (M_r 43000), bovine serum albumin (M_r 67000), pig M_4 lactate dehydrogenase (M_r 140000) and bovine liver catalase (M_r 248000). A 3mg portion of each protein was applied to the column in 0.75ml of buffer and eluted at 25ml/h to give 2.5ml fractions. The SCAD M_r was interpolated on a plot of elution volume against $\log M_r$.

The subunit M_r was estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. A 10μg portion of each of the following standards was run on a 15%-acrylamide gel after denaturation by heating in 5% (w/v) sodium dodecyl sulphate and 2% (v/v) mercaptoethanol: pig M_4 lactate dehydrogenase (M_r 35000), yeast alcohol dehydrogenase (M_r 41000), ovalbumin (M_r 45000), bovine glutamate dehydrogenase (M_r 56000) and bovine serum albumin (M_r 67000). A 10μg portion of similarly treated SCAD was run on the same gel, and the subunit M_r was interpolated from a plot of relative mobility against $\log M_r$.

Results and discussion

Purification

All operations were carried out at 4°C and all phosphate buffers contained 0.3mM-EDTA. The crude protein extract from the mitochondria (typically about 1 litre) was fractionated with $(NH_4)_2SO_4$. Protein precipitated between 40% (242g/litre) and 57% (a further 110g/litre original volume) $(NH_4)_2SO_4$ saturation was resuspended in a minimal volume of 25mM-potassium phosphate buffer, pH7.6 containing 1mM-phenylmethanesulphonyl fluoride and dialysed overnight against 10 litres of the same buffer without the proteinase inhibitor. The non-diffusible material was applied to a column of DEAE-Sephadex A-50 equilibrated in the dialysis buffer. The elution profile (Fig. 1) shows complete separation of SCAD from GAD and long-chain acyl-CoA dehydrogenase, in concordance with similar findings by Beinert (1962) and Davidson & Schulz (1982). This simple and complete separation makes ox liver a good enzyme source.

Fractions were pooled, according to their activity towards butyryl-CoA and their visible- and u.v.-absorption spectra (fractions should have a well-defined absorption peak at about 445nm and an A_{270}/A_{455} ratio of 22:1 at most), and concentrated by dialysis against 2 litres of 15% poly(ethylene glycol) (M_r 20000) dissolved in 25mM-potassium phosphate buffer, pH7.6. The yellow–green solution thus obtained had a visible-absorption spectrum similar to that of the purified enzyme, and the A_{270}/A_{445} ratio and the specific activity indicated the SCAD to be about 30% pure.

The most effective further purification was obtained by using Pharmacia chromatofocusing materials. The elution profile (Fig. 2*a*) shows a complicated pattern of elution of SCAD activity, the three main features being a leading edge at about pH5.3 and two other peaks at pH5.12 and

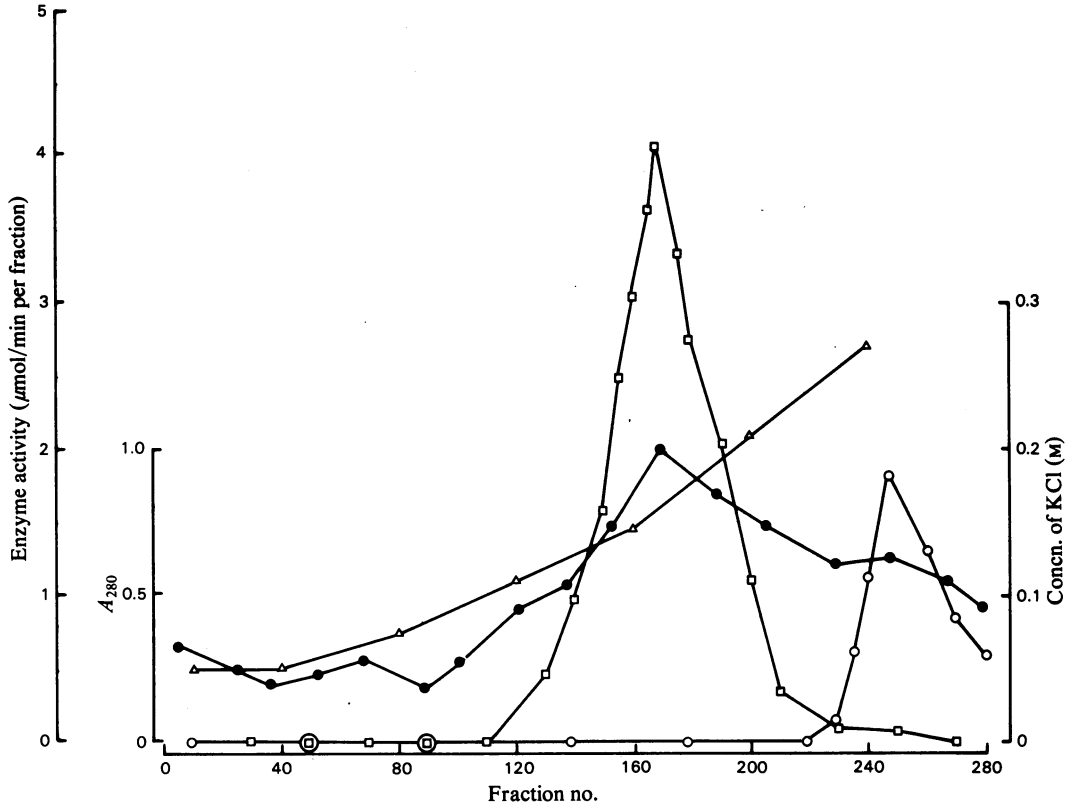

Fig. 1. *Elution of SCAD from DEAE-Sephadex A-50*

Protein precipitated by 40–57% saturation with $(NH_4)_2SO_4$ was desalted and applied to a 5cm × 20cm column of DEAE-Sephadex A-50 previously equilibrated in 25mM-potassium phosphate buffer, pH7.6. The column was washed with 50mM-KCl, and developed with a 1600ml gradient of KCl (△) up to 0.3M at a flow rate of 90ml/h; 6ml fractions were collected. The absorbance at 280nm (●) and the enzyme activity towards butyryl-CoA (□) (50μM) and dodecanoyl-CoA (○) (30μM) were monitored.

5.0. Spectral differences between the SCAD forms under each peak could be observed. Earlier fractions (e.g. fraction 25) had no long-wavelength absorbance, but a progressive increase in the A_{685}/A_{445} ratio was observed in later, greener, fractions.

The long-wavelength absorption centred at 685nm was presumed to be due to a charge-transfer interaction between the enzyme-bound FAD and a tightly bound CoA persulphide (Williamson *et al.*, 1982*a*). This green form of SCAD can be converted into the unliganded yellow form by prolonged reduction with $Na_2S_2O_4$ (which destroys CoA persulphide) followed by extensive re-oxidation (Steyn-Parvé & Beinert, 1958*b*; Engel & Massey, 1971*b*).

Chromatofocusing of SCAD (after DEAE-Sephadex chromatography), de-greened by overnight dialysis against 10mM-$Na_2S_2O_4$ in 100mM-potassium phosphate buffer, pH7.6, and re-oxidized by further dialysis in histidine buffer, gave a single peak of activity at pH5.3 (Fig. 2*b*).

This finding that differently liganded forms of SCAD possess different isoelectric points on chromatofocusing could reflect changes in the surface properties of the protein upon binding the CoA persulphide or may be due simply to the extra negative charge introduced on to the enzyme. The possibility that different isoenzymic forms of SCAD cause the multiple chromatofocusing peaks is therefore ruled out. This is in agreement with Seeley & Holmes (1981), who found no SCAD isoenzymes in the mouse.

The SCAD fractions, from chromatofocusing, having an A_{270}/A_{450} ratio of 5.5:1 were pooled, although to obtain a good yield fractions with ratios up to 6.0 could be included with little detriment to purity. The enzyme gave one band with 22μg load-

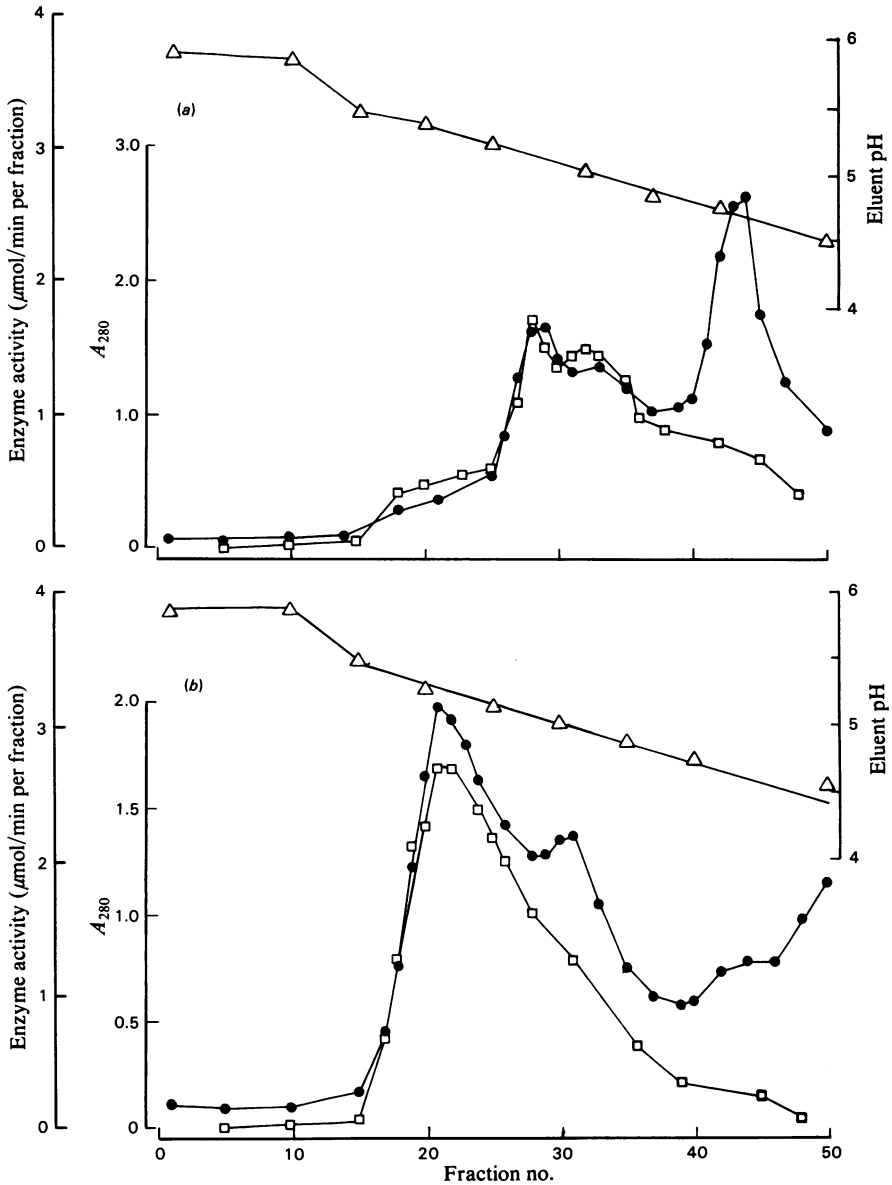

Fig. 2. *Chromatofocusing on PBE 94 resin of (a) SCAD from the DEAE-Sephadex step and (b) de-greened SCAD from the DEAE-Sephadex step*

(*a*) The concentrated eluate from DEAE-Sephadex A-50 was dialysed against 25mM-histidine buffer, pH6.3, and applied to a column (1cm × 24cm) of PBE 94 resin equilibrated in the same buffer. The column was developed with a $\frac{1}{8}$ dilution of Polybuffer 74, pH4.0, at a flow rate of 22ml/h, and 1.8ml fractions were collected (Pharmacia, 1981). Eluent pH (△), absorbance at 280nm (●) and enzyme activity (□) were monitored. (*b*) The concentrated eluate from DEAE-Sephadex A-50 was de-greened by overnight dialysis against 10mM-$Na_2S_2O_4$ in 100mM-potassium phosphate buffer, pH7.6, and re-oxidized by dialysis against 25mM-histidine buffer, pH6.3. Chromatofocusing conditions and symbols were as for (*a*).

ing on sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis, although several very faint bands were visible at higher loadings. Table 1 shows the purification scheme.

M_r

The subunit M_r was estimated by sodium dodecyl sulphate/polyacrylamide-gel electro-

Table 1. *Purification of ox liver SCAD*

Amounts of protein were measured by the Bradford (1978) method. Total enzyme activity was measured with 50 μM-butyryl-CoA as described in the Materials and methods section.

Fraction	Total protein (mg)	Total activity (μmol of butyryl-CoA/min)	Specific activity (μmol/min per mg of protein)	Purification factor	Yield (%)
Crude mitochondrial extract	4165	180	0.043	1	100
40–57%-satn.-$(NH_4)_2SO_4$ fraction	2770	178	0.064	1.49	99
DEAE-Sephadex eluate	296	119	0.4	9.3	66
Chromatofocusing eluate	27	40	1.42	33	22

Table 2. *Comparison of spectral parameters for the pure forms of ox liver SCAD obtained here with those previously reported*

Enzyme form	Absorbance maxima (nm)	Relative absorbances
Green (Mahler, 1954)	255, 355, 432.5, 685	7.75:0.86:1:0.25
Green (present work)	270, 362, 444, 685	6.3:0.89:1:0.035
Yellow (present work)	270, 368, 448	5.5:0.81:1

phoresis as 41000, and this compares well with the value of 42000 obtained by ultracentrifugation in the presence of guanidinium chloride (Murfin, 1974).

The M_r of the native enzyme was determined as 162000 by using a calibrated Sephadex G-150 column. A tetrameric structure, with four presumably identical subunits, is inferred. The quaternary structure and subunit size thus appear similar for all acyl-CoA dehydrogenases so far studied (Engel & Massey, 1971*a*; Hall *et al.*, 1976; Thorpe *et al.*, 1979).

Spectral properties

Fig. 3 shows the absorption spectra of native green and yellow forms of purified SCAD. The latter spectrum is clearly that of a flavoprotein, and the prosthetic group has previously been identified as FAD (Mahler, 1954). There are, however, quite marked spectral differences between our green preparation and the much greener Mahler (1954) preparation, as judged by the $A_{685}/A_{432.5}$ ratio, as reported in Table 2. Our observations suggest that in its green form the enzyme is much more stable, and it is possible that the greater greenness of earlier preparations was due to selective removal of the yellow form of the enzyme by the lengthy, harsh, procedures used.

The visible-absorbance maximum of free FAD occurs at 450nm, with an absorption coefficient of 11.3mM^{-1}·cm^{-1}. However, the FAD bound to yellow SCAD has an absorption coefficient of 14.9mM^{-1}·cm^{-1}, as determined by release with either guanidinium chloride or trichloroacetic acid. Although enhancement of the main visible-region flavin and on binding to various apoproteins is frequently observed, this absorption coefficient is exceptionally high. It falls in line, however, with values of 15.4mM^{-1}·cm^{-1} reported by Thorpe *et al.* (1979) for pig kidney GAD and 14.4mM^{-1}·cm^{-1} for the SCAD from *M. elsdenii* (Williamson & Engel, 1984).

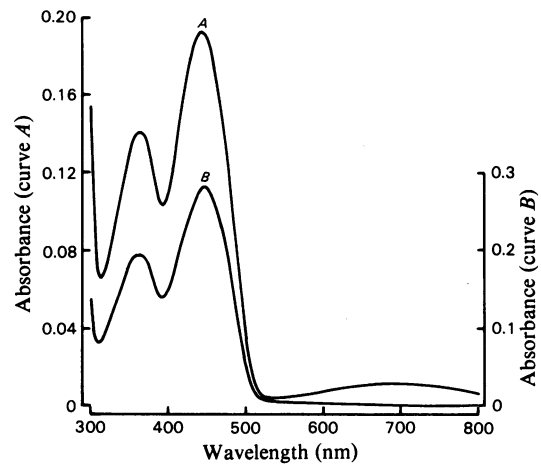

Fig. 3. *Absorption spectra of purified native forms of SCAD*

The Figure shows the absorption spectra of the green form (curve *A*) and the yellow form (curve *B*) of SCAD, both in 50mM-potassium phosphate buffer, pH 7.6, and prepared as described in the text. Note the different vertical scales employed to avoid overlap.

Spectral perturbations induced by addition of certain CoA-containing ligands

In all of these experiments the enzyme was first de-greened by a 14h anaerobic dialysis against 10mM-$Na_2S_2O_4$ in 100mM-potassium phosphate buffer, pH7.6, followed by re-oxidation by dialysis against 50mM-potassium phosphate buffer, pH7.6, for 6h.

(i) *Addition of substrates.* As with other mammalian acyl-CoA dehydrogenases, the aerobic titration with substrate gave rise to partial reduction of the flavin and the appearance of a long-wavelength absorption band centred at 560nm (Fig. 4). Detailed investigations into the nature of such acyl-CoA dehydrogenase–substrate complexes have been previously confined to the pig GAD, and show that an FAD–substrate-anion charge-transfer interaction occurs (Hall *et al.*, 1979; Frerman *et al.*, 1980; Thorpe *et al.*, 1981; Schmidt *et al.*, 1981). This complex remained stable for several hours in our experiments.

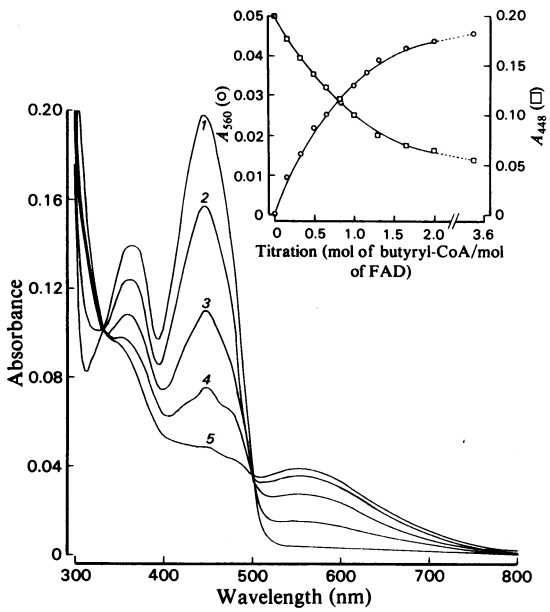

Fig. 4. *Aerobic titration of de-greened SCAD with butyryl-CoA*

Enzyme (13.4nmol, by flavin) in 1ml of 50mM-potassium phosphate buffer, pH7.6, at 6°C was titrated with 5–10μl additions of butyryl-CoA. Spectra were recorded immediately after additions. Spectra were not corrected for the slight dilution, but absorbances for the titration plot were corrected (see inset). The additions were as follows: 0 (curve *1*), 0.33 (curve *2*), 0.82 (curve *3*), 1.3 (curve *4*) and 3.6 (curve *5*)mol of butyryl-CoA/mol of flavin.

(ii) *Addition of CoA persulphide.* A mixture containing CoA persulphide was generated by addition of a 30-fold excess of Na_2S to 1mg of CoA in 1ml of 50mM-potassium phosphate buffer, pH7.6 (Williamson *et al.*, 1982*a*). After incubation at room temperature for 3h, portions were added to 12.8nmol of enzyme (by flavin content) until no further spectral changes occurred. Excess of CoA persulphide was removed by dialysis. Fig. 5 shows the spectrum of enzyme fully liganded with CoA persulphide. The main flavin peaks were blue-shifted to 430 and 357nm, with the appearance of a long-wavelength band at 685nm. The A_{685}/A_{430} ratio was 0.29:1, and the absorption coefficient at 430nm was 12.2mM^{-1}·cm^{-1}. In the case of *M. elsdenii* SCAD the fully persulphide-liganded form has absorbance maxima at 430 and 710nm with an A_{710}/A_{430} ratio of 0.54:1 and an absorption coefficient of 10.4mM^{-1}·cm^{-1} (Engel, 1981).

(iii) *Addition of acetoacetyl-CoA.* The addition of acetoacetyl-CoA to the enzyme gave rise to changes shown in Fig. 6. The main flavin absorbance maximum was strongly blue-shifted to 425nm and a long-wavelength absorption band appeared at 555nm. A very similar complex with acetoacetyl-CoA was first described for *M. elsdenii* SCAD by Engel & Massey (1971*b*), who pointed out the likely regulatory significance if similar complexes were formed by mammalian acyl-CoA dehydrogenases. Since then acetoacetyl-CoA com-

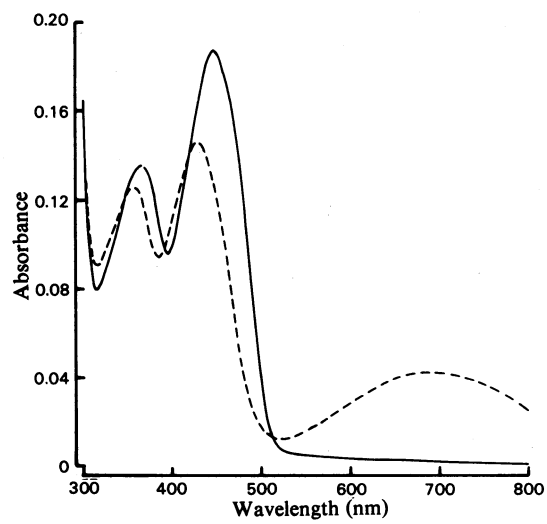

Fig. 5. *Absorption spectra of de-greened and fully-green SCAD*

De-greened enzyme (12.8nmol, by flavin) in 1ml of 50mM-potassium phosphate buffer, pH7.6 (——), was treated with an excess of CoA persulphide and dialysed (----) to give the spectrum shown.

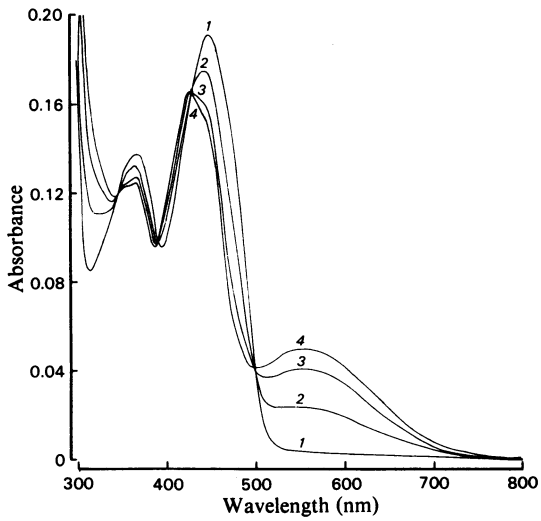

Fig. 6. *Aerobic titration of de-greened SCAD with acetoacetyl-CoA*

Enzyme (12.8nmol, by flavin) in 1ml of 50mM-potassium phosphate buffer, pH7.6, was titrated with 5–10μl additions of acetoacetyl-CoA, and spectra were recorded immediately. Spectra were not corrected for the slight dilution. The additions were as follows: 0 (curve *1*), 0.47 (curve *2*), 0.93 (curve *3*) and 1.25mol (curve *4*) of acetoacetyl-CoA/mol of flavin.

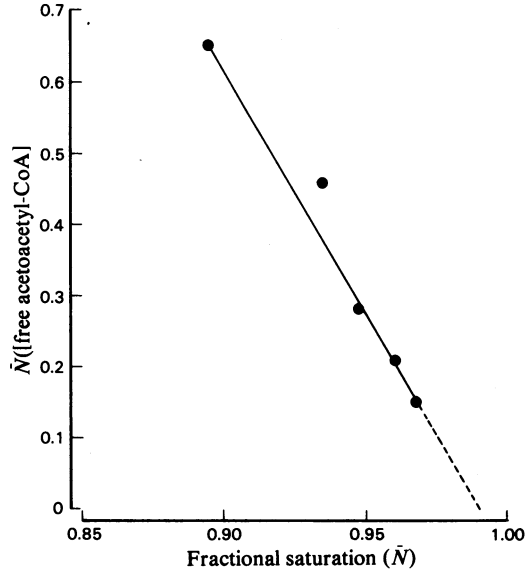

Fig. 7. *Aerobic quantitative titration of de-greened SCAD with acetoacetyl-CoA*

Enzyme (27.7nmol, by flavin) in 1ml of 50mM-potassium phosphate buffer, pH7.6, was titrated with 5–10μl additions of 0.4mM-acetoacetyl-CoA. A_{555} was recorded immediately and corrected for dilution. A Scatchard plot of appropriate data is shown. The dissociation constant calculated from the slope of this graph was 0.15μM.

plexes have been reported for the pig GAD (Benecky *et al.*, 1979), and substantial evidence exists to suggest that these are charge-transfer complexes (Williamson *et al.*, 1982*b*). It is now of interest that mammalian SCAD also forms such a complex. Experiments on impure SCAD (Davidson & Schulz, 1982) and purified SCAD (L. Shaw, unpublished work) show that acetoacetyl-CoA is a potent competitive inhibitor, with a K_i of about 1μM.

Acetoacetyl-CoA bound to the enzyme with a stoicheiometry of 1mol/mol of FAD and a dissociation constant of 0.15μM (Fig. 7).

Kinetic properties

(i) *pH–activity profile.* The standard dichlorophenol-indophenol-reduction assay was used to test the dependence of activity towards butyryl-CoA (50μM) on pH. Activity was measured over a range of pH (pH6–10) in a potassium phosphate/Tris/glycine (each at 40mM) buffer adjusted to the desired pH with NaOH or HCl. A shallow pH–activity profile was obtained, with peak activity at pH7.1. Rates at pH6 and 10 were 70% and 48% respectively of the maximum value. The same pH optimum was obtained in 120mM-potassium phosphate buffers over a narrower range, again for 50μM-butyryl-CoA.

(ii) *Substrate specificity.* The kinetic parameters of SCAD for several straight-chain acyl-CoA substrates were determined. With all substrates linear Lineweaver–Burk and Hanes plots were obtained, from which K_m and $V_{max.}$ values could be determined. Fig. 8 shows the $V_{max.}$ and K_m profiles. Surprisingly, maximal activity was obtained with pentanoyl-CoA. However, butyryl-CoA showed the lowest K_m. An activity profile presented by Green *et al.* (1954) for fixed acyl-CoA concentrations gave an activity maximum with butyryl-CoA. In similar studies, both the sheep and rat liver SCAD (Beinert, 1963; Furuta *et al.*, 1981) and the *M. elsdenii* SCAD (Williamson, 1983) gave optimal activity with butyryl-CoA. The rat enzyme, however, was tested only with even-chain acyl-CoA substrates.

Very little information has been reported on the rate of propionyl-CoA turnover by SCAD. Despite the considerable rate obtained by Green *et al.* (1954), we obtained very low rates with propionyl-CoA ($V_{max.}$ = 0.5μmol/min per μmol of FAD).

In summary, the three-step purification pre-

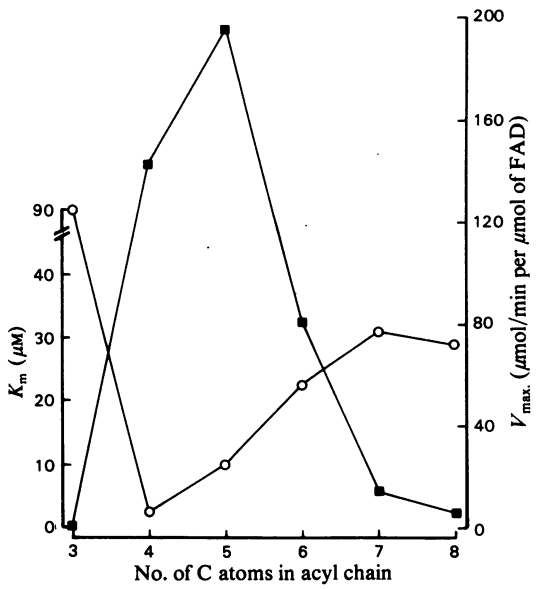

Fig. 8. *Substrate-specificity profile of SCAD*
The small (often negligible) background decrease in A_{600} measured on addition of enzyme to substrate and 0.001% dichlorophenol-indophenol in 120mM-potassium phosphate buffer, pH 7.1 (25°C), was subtracted from the full rate observed on addition of 15μl of phenazine ethosulphate to a final concentration of 0.6mM. K_m (○) and $V_{max.}$ (■) parameters were estimated by eye from Lineweaver–Burk plots. Progress curves at high substrate concentrations were linear, but at lower concentrations curvature was apparent. Rates were measured in duplicate and were all within 5% error (and 10% error for rates with propionyl-CoA). Each plot consisted of at least five data points.

sented here yields sufficient quantities of mammalian SCAD, from manageable amounts of tissue, for detailed mechanistic studies. The enzyme is shown to be structurally similar to a bacterial SCAD and to other mammalian acyl-CoA dehydrogenases. In common with other mammalian acyl-CoA dehydrogenases this enzyme forms an enzyme–substrate charge-transfer complex in which the reduced flavin is protected from O_2. Comparative studies between this enzyme and that from the anaerobic bacterium *M. elsdenii*, which allows rapid reoxidation of its substrate-reduced flavin, could yield insight into the role of this complex in catalysis.

The mammalian SCAD interacts with other CoA-containing compounds to form complexes that are spectrally similar to those of the bacterial enzyme and GAD. In particular, it is clear that the long-wavelength band at 685nm in preparations of SCAD from ox liver, like the corresponding band at 710nm in the bacterial enzyme, is caused by a charge-transfer interaction with CoA persulphide. It is not yet clear whether this complex is an artifact of the purification procedure or whether it has some regulatory significance. Certainly, the tight binding of acetoacetyl-CoA by mammalian SCAD now makes this appear a plausible point for the control of β-oxidation by one of the end products of the process.

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References

Armstrong, J. McD. (1964) *Biochim. Biophys. Acta* **86**, 194–197
Beinert, H. (1957) *J. Biol. Chem.* **225**, 465–478
Beinert, H. (1962) *Methods Enzymol.* **5**, 546–557
Beinert, H. (1963) *Enzymes 2nd Ed.* **7**, 447–466
Benecky, M., Li, T. Y., Schmidt, J., Frerman, F., Watters, K. L. & McFarland, J. (1979) *Biochemistry* **18**, 3471–3476
Bradford, M. M. (1978) *Anal. Biochem.* **72**, 248–254
Crane, F. L. & Beinert, H. (1956) *J. Biol. Chem.* **218**, 717–731
Crane, F. L., Glen, J. L. & Green, D. E. (1956*a*) *Biochim. Biophys. Acta* **22**, 475–486
Crane, F. L., Mii, S., Hauge, J. G., Green, D. E. & Beinert, H. (1956*b*) *J. Biol. Chem.* **218**, 701–716
Davidson, B. & Schulz, H. (1982) *Arch. Biochem. Biophys.* **213**, 155–162
Engel, P. C. (1981) *Methods Enzymol.* **71**, 359–366
Engel, P. C. & Massey, V. (1971*a*) *Biochem. J.* **125**, 879–887
Engel, P. C. & Massey, V. (1971*b*) *Biochem. J.* **125**, 889–902
Frerman, F. E., Miziorko, H. M. & Beckman, J. D. (1980) *J. Biol. Chem.* **255**, 11192–11198
Furuta, S., Miyazawa, S. & Hashimoto, T. (1981) *J. Biochem. (Tokyo)* **90**, 1739–1750
Green, D. E., Mii, S., Mahler, H. R. & Bock, R. M. (1954) *J. Biol. Chem.* **206**, 1–12
Hall, C. L. & Kamin, H. (1975) *J. Biol. Chem.* **250**, 3476–3486
Hall, C. L., Heijkenskjöld, L., Bártfai, T., Ernster, L. & Kamin, H. (1976) *Arch. Biochem. Biophys.* **177**, 402–414
Hall, C. L., Lambeth, D. J. & Kamin, H. (1979) *J. Biol. Chem.* **254**, 2023–2031
Hauge, J. G., Crane, F. L. & Beinert, H. (1956) *J. Biol. Chem.* **219**, 727–733
Hoskins, D. D. (1966) *J. Biol. Chem.* **241**, 4472–4479
Kawaguchi, A., Yoshimura, T. & Okuda, S. (1981) *J. Biochem. (Tokyo)* **89**, 337–339
Mahler, H. R. (1954) *J. Biol. Chem.* **206**, 13–26
Massey, V. & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci.* **227**, 446–465
Mayhew, S. G. & Massey, V. (1969) *J. Biol. Chem.* **244**, 794–802
Michal, G. & Bergmeyer, H.-U. (1963) in *Methods in Enzymatic Analysis* (Bergmeyer, H.-U., ed.), p. 512, Verlag Chemie, Weinheim

Murfin, W. W. (1974) Ph.D. Thesis, Washington University
Noda, C., Rhead, W. J. & Tanaka, K. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2646–2650
Pharmacia (1981) *Information Booklet: Chromatofocusing with Polybuffer and PBE*, Pharmacia, Uppsala
Schmidt, J., Reinsch, J. & McFarland, J. T. (1981) *J. Biol. Chem.* **256**, 11667–11670
Seeley, T.-L. & Holmes, R. S. (1981) *Biochem. Genet.* **19**, 333–345
Seubert, W. & Lynen, F. (1953) *J. Am. Chem. Soc.* **75**, 2787
Simon, E. J. & Shemin, D. (1953) *J. Am. Chem. Soc.* **75**, 2520
Stadtman, E. R. (1954) *Methods Enzymol.* **3**, 931–941
Stern, J. R. (1956) *J. Biol. Chem.* **221**, 33–44
Steyn-Parvé, E. P. & Beinert, H. (1958*a*) *J. Biol. Chem.* **233**, 843–852
Steyn-Parvé, E. P. & Beinert, H. (1958*b*) *J. Biol. Chem.* **233**, 853–861
Thorpe, C., Matthews, R. G. & Williams, C. H., Jr. (1979) *Biochemistry* **18**, 331–337
Thorpe, C., Ciardelli, T. L., Stewart, C. J. & Wieland, T. (1981) *Eur. J. Biochem.* **118**, 279–282
Williamson, G. (1983) Ph.D. Thesis, University of Sheffield
Williamson, G. & Engel, P. C. (1984) *Biochem. J.* **218**, 521–529
Williamson, G., Engel, P. C., Mizzer, J. F., Thorpe, C. & Massey, V. (1982*a*) *J. Biol. Chem.* **257**, 4314–4320
Williamson, G., Engel, P. C., Nishina, Y. & Shiga, K. (1982*b*) *FEBS Lett.* **138**, 29–32