The suicide inactivation of ox liver short-chain acyl-CoA dehydrogenase by propionyl-CoA

Formation of an FAD adduct

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1. Propionyl-CoA gave an unexpectedly low turnover (0.03 s^{-1}) and high K_m ($153\text{ }\mu\text{M}$) as a substrate for ox liver short-chain acyl-CoA dehydrogenase (SCAD). 2. On addition of an excess of propionyl-CoA to SCAD the flavin A_{448} decreased to about 30% of its original value and the peak at 368 nm was replaced by one at 335 nm. The decrease in A_{448} exhibited first-order kinetics and correlated with a first-order decrease in the enzyme's catalytic activity to 22% of the initial value. 3. The flavin, released from propionyl-CoA-treated enzyme with trichloroacetic acid, reacted with O_2 to form a stable free radical. This suggests that a reduced N-5 flavin adduct is formed on the enzyme and protected from O_2 . The released adduct was separated from unmodified flavin and excess propionyl-CoA by h.p.l.c., and was shown by ^3H -labelling to contain CoA. 4. The incompleteness of the decrease in the enzyme's A_{448} and specific activity on incubation with propionyl-CoA probably reflects an equilibrium between covalently and non-covalently bound acyl-CoA, since the spectral changes could be reversed. The enzyme was also re-activated by dilution and incubation with a large molar excess of butyryl-CoA. The rate constant, approx. $2 \times 10^{-3}\text{ s}^{-1}$, for re-activation, taken with the extrapolated rate constant for the opposing inactivation reaction, $8.9 \times 10^{-3}\text{ s}^{-1}$, explains the 22% residual activity at equilibrium. 5. The results suggest that propionyl-CoA is a suicide inhibitor for SCAD.

The acyl-CoA dehydrogenases are a group of FAD-linked enzymes that catalyse the introduction of the $\alpha\beta$ -double bond into acyl-CoA substrates during β -oxidation (Seubert & Lynen, 1953). Three straight-chain acyl-CoA dehydrogenases, differing in their acyl chain-length specificity (Hauge *et al.*, 1956; Crane *et al.*, 1956; Green *et al.*, 1954), function in the β -oxidation pathway for fatty acids with chain lengths greater than C_3 (Greville & Tubbs, 1968). By contrast, propionyl-CoA, derived from odd-chain fatty acids, certain amino acids and dietary propionic acid, is carboxylated and then oxidized via the methylmalonyl-CoA pathway (Kaziro & Ochoa, 1964). It has also been suggested that propionyl-CoA can be β -oxidized via acryloyl-CoA (Rendina & Coon, 1957), but this is now thought unlikely (Kaziro &

Ochoa, 1964; Vagelos, 1960), even though, in the first publication on SCAD, a substantial rate was reported for propionyl-CoA (Green *et al.*, 1954). Using a more reliable assay, Shaw & Engel (1984) demonstrated that k_{cat} for propionyl-CoA with SCAD was about 1.2% that for butyryl-CoA. This suggests that β -oxidation of propionyl-CoA by this enzyme is unlikely to occur *in vivo*.

This 80-fold difference between the values of k_{cat} for propionyl-CoA and butyryl-CoA poses intriguing questions concerning the nature of enzyme-substrate interactions required for catalysis by SCAD. Possibly the anchoring and orientation of propionyl-CoA is too imprecise to allow efficient catalysis. Frerman *et al.* (1980) reported only weak interaction between pig general acyl-CoA dehydrogenase and propionyl-CoA and concluded that the acyl group provides a significant proportion of the free energy of binding. Auer & Frerman (1980) reported a re-orientation of the

Abbreviation used: SCAD, short-chain acyl-CoA dehydrogenase.

flavin in general acyl-CoA dehydrogenase upon substrate binding and suggested a substrate-induced conformational change. Possibly propionyl-CoA is unable to elicit a similar activating conformational change in an induced-fit mechanism (Koshland, 1958) for SCAD.

In the present paper we provide a kinetic, spectroscopic and chemical analysis of the interaction of propionyl-CoA with SCAD. It emerges that propionyl-CoA, though a poor substrate, is by no means an inert ligand for this enzyme. The possible relevance of the results to the catalytic mechanism of SCAD is discussed.

Materials and methods

Enzyme preparation

SCAD was purified from fresh ox liver (Shaw & Engel, 1983, 1984) and, unless stated otherwise, used in the de-greened form (Williamson & Engel, 1982) in 50 mM-potassium phosphate buffer, pH 7.6.

Acyl-CoA synthesis and purification

Propionyl-CoA and butyryl-CoA were prepared from acid anhydrides (Simon & Shemin, 1953; Stadtman, 1957). Acetoacetyl-CoA was synthesized by the method of Michal & Bergmeyer (1963). Acryloyl-CoA was made by adding acryloyl chloride dissolved in redistilled tetrahydrofuran to a solution of CoA in 0.2 M-KHCO₃, pH 7.5–8.0. After extraction of fatty acids with diethyl ether, the acyl-CoA compounds were freeze-dried, resuspended in a minimal volume of cold water and purified by h.p.l.c. (Applied Chromatography Systems, Luton, Beds., U.K.) on a 250 mm × 8 mm Spherisorb 5 ODS 2 (C₁₈) column (Hichrom, Reading, Berks., U.K.) by using a gradient of 3.5–70% (v/v) methanol in 10 mM-KH₂PO₄ (Corkey *et al.*, 1981).

Pentanoyl-CoA, hexanoyl-CoA, heptanoyl-CoA and octanoyl-CoA were synthesized by the method of Kawaguchi *et al.* (1981), and purified on a column of DEAE-Sephadex A-25 (Williamson & Engel, 1984).

Enzyme assay

The dye-reduction assay method described by Shaw & Engel (1984) was used in routine specific-activity assays with 100 μM-butyryl-CoA.

For kinetic measurements at 25°C with propionyl-CoA, 0.2 M-potassium phosphate, pH 7.6, was the buffering medium, and 2,6-dichlorophenol-indophenol and *N*-ethylphenazinium ethosulphate concentrations were the same as in the

assay with butyryl-CoA. The enzyme was added last.

Reagents

Chemicals were from the following suppliers: butyric anhydride and propionic anhydride, 2,6-dichlorophenol-indophenol, guanidinium chloride (AristaR grade) and trichloroacetic acid from BDH Chemicals, Atherstone, Warwicks., U.K.; acryloyl chloride and ²H₂O from Fluorochem, Glossop, Derbys., U.K.; diketene from Aldrich Chemical Co., Poole, Dorset, U.K.; methanol (h.p.l.c. grade), tetrahydrofuran and Na₂S₂O₄ from Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Instrumentation

All absorbances, spectra and enzymic reaction rates were measured in the thermostatically controlled cuvette holder of a Cary 219 spectrophotometer (Varian Associates, Walton-on-Thames, Surrey, U.K.). Quartz cuvettes (1 ml) were used wherever possible. Spectra under anaerobic conditions, however, were recorded with the use of a glass anaerobic cell designed and built in the Sheffield department. This, a modification of that described by Foust *et al.* (1969), consisted of an upper titrant reservoir connected via a calibrated capillary to the optical cuvette below. Anaerobic conditions were achieved by repeated cycles of vacuum degassing and equilibration with O₂-free N₂. As an added precaution, further O₂ scavenging was achieved by addition of glucose and glucose oxidase to the contents of the cuvette.

E.p.r. spectra were measured aerobically, at room temperature and at liquid-N₂ temperature, with a Varian E.9 e.s.r. spectrometer interfaced with the University of Sussex DEC VAX 11/780 computer and a DEC PDP 11/10 computer. Samples (100 μl) were placed in calibrated quartz tubes.

Liquid-scintillation counting

[G-³H]CoA (specific radioactivity 1.2 Ci/mmol), purchased from New England Nuclear, Southampton, U.K., was diluted to a specific radioactivity of 3.25 Ci/mol with unlabelled CoA and converted into propionyl-[G-³H]CoA with propionic anhydride.

Liquid-scintillation counting was performed with a PW 4700 counter (Philips, Cambridge, U.K.). The radioactivities of 1 ml aqueous samples were counted in 10 ml of Scintran cocktail T (BDH Chemicals). Counting was corrected with a pre-programmed quench curve for tritium, constructed by using the external channels-ratio method. Counting efficiency was generally about 30%.

H.p.l.c. of flavins extracted from SCAD

Extracted flavins were separated by using the same method as employed for acyl-CoA purification, except that an analytical (250 mm $\times$ 5 mm) Spherisorb 5 ODS 2 (C_{18}) column was used.

Results*Kinetics of propionyl-CoA oxidation by SCAD in the dye-reduction assay*

Rate values were collected for a 33–500 μ M range of propionyl-CoA concentration. The apparent K_m and k_{cat} for propionyl-CoA oxidation were estimated as 153 μ M ($\pm 6.1 \mu$ M) and 0.03 s^{-1} ($\pm 0.0005 \text{ s}^{-1}$) respectively by computer-fitting of the data to a Michaelis–Menten plot (Hoy & Goldberg, 1971). The corresponding K_m and k_{cat} for butyryl-CoA are 2.7 μ M and 2.5 s^{-1} respectively (Shaw & Engel, 1984).

Although its binding constant was not directly measured, propionyl-CoA up to 20 μ M was unable to inhibit SCAD competitively in a catalytic assay, even with butyryl-CoA concentrations as low as 2 μ M.

The results indicate that propionyl-CoA interacts with SCAD in a much weaker and catalytically less favourable manner than does butyryl-CoA.

Effect of propionyl-CoA on enzyme activity

During the catalytic assays with propionyl-CoA as substrate, the reaction traces showed marked curvature, which could not be adequately explained by depletion of reactants or accumulation of products. In a typical assay with 200 μ M-propionyl-CoA, the rate of 2,6-dichlorophenol-indophenol reduction decreased to about 13% of its initial value after 6 min; in this time the concentration of 2,6-dichlorophenol-indophenol and therefore also of propionyl-CoA had declined by 4 μ M, as compared with the initial values of 28 μ M and 200 μ M respectively. At this point a second addition of enzyme, equal to the first, increased the rate to a value roughly equal to the sum of the previous initial and final rates. Evidently the initial addition of enzyme had largely been inactivated, even though SCAD is normally stable under the buffer conditions used.

To test this hypothesis, 6.7 μ M-SCAD (by flavin) was incubated with 200 μ M- and 500 μ M-propionyl-CoA in 200 mM-potassium phosphate buffer, pH 7.6, at 25°C. Samples (10 μ l) were withdrawn periodically for assays of catalytic activity. A steady decline in activity to 22% of the initial value was observed for both propionyl-CoA concentrations. Plots of $\ln[A_0 - A_e]/(A_t - A_e)$ versus time were linear (A_0 being activity before propionyl-

CoA addition, A_e the final activity and A_t the activity at time t) (Fig. 1) and gave apparent first-order rate constants ($k_{app.}$) of 5.6×10^{-3} and $7.2 \times 10^{-3} \text{ s}^{-1}$.

Final levels of approx. 22% residual activity were obtained with a wide range of propionyl-CoA concentrations. In order to achieve lesser extents of inactivation very low concentrations were used; thus in experiments with 0.3 μ M-SCAD (by flavin) final levels of 83%, 57% and 42% activity respectively were obtained with initial propionyl-CoA concentrations of 0.3 μ M, 0.6 μ M and 2 μ M.

Labelling of SCAD with radioactive propionyl-CoA

Incubation of SCAD with various concentrations of propionyl-[G- 3 H]CoA, followed by removal of excess thioester by protein precipitation and washing with a saturated solution of $(\text{NH}_4)_2\text{SO}_4$, resulted in a linear relationship between the amount of bound radioactivity and loss in enzyme activity (Fig. 2). The stoichiometry was consistent with incorporation of approx. 1 mol of CoA/mol of inactivated flavin.

Addition of a stoichiometric amount of butyryl-CoA together with a 5-fold excess of propionyl-CoA to SCAD at a final concentration of 6.6 μ M (by flavin) gave only a 13% loss of activity, as compared with 78% in the absence of butyryl-CoA.

The results show that propionyl-CoA binds at the active site of SCAD, causing a pseudo-first-order inactivation accompanied by a strong association of propionyl-CoA, or a derived chemical species, with the enzyme.

Fig. 1. Semi-logarithmic plot of the relative decrease in activity of propionyl-CoA-treated SCAD against time. SCAD (6.7 μ M by flavin) was incubated with 500 μ M- (○) and 200 μ M- (●) propionyl-CoA at 25°C in 200 mM-potassium phosphate buffer, pH 7.6. Samples (10 μ l) were removed and immediately assayed for SCAD activity with 100 μ M-butyryl-CoA.

Spectral investigations into the interaction of propionyl-CoA with SCAD

Aerobic addition of acyl-CoA substrates to SCAD instantaneously produces a relatively stable complex with decreased A_{448} and a charge-transfer band present at about 560nm (Mahler, 1954; Steyn-Parvé & Beinert, 1958; Shaw & Engel,

Fig. 2. Relationship between SCAD activity and enzyme-bound propionyl-[G- 3 H]CoA

SCAD (9.1 nmol, by flavin), in 0.3 ml, was incubated with 0, 2.7, 4.5, 9.1, 18.2, 27.3 and 45 nmol of propionyl-[G- 3 H]CoA at 22°C for 20 min. To remove excess propionyl-CoA, 0.9 ml of a cold saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added. After mixing for 15 s, the enzyme in each incubation was precipitated by a 10 min centrifugation at 9000g at 4°C. The pellets were resuspended in 1 ml of 70%-saturated $(\text{NH}_4)_2\text{SO}_4$ in 50 mM-potassium phosphate buffer, pH 7.6, again at 4°C. The second wash was repeated and the final pellets were resuspended in 1 ml of 50 mM-potassium phosphate buffer, pH 7.6. The specific activity of each final pellet was measured, and the extent of incorporation of propionyl-[G- 3 H]CoA was determined by liquid-scintillation counting.

1984). The extent of these spectral changes was monitored for a series of acyl-CoA substrates and increased with k_{cat} , determined in the catalytic assay (Table 1). Propionyl-CoA might therefore have been expected to give slight flavin reduction and a charge-transfer band of low intensity. In fact a 5-fold molar excess of propionyl-CoA (over flavin) gave slow and extensive flavin bleaching (70%), no charge-transfer band was seen and a new peak appeared at 335 nm (Fig. 3). A single isosbestic point was present at 343 nm. Addition of more propionyl-CoA gave no further changes. The resultant spectrum was apparently stable for an hour, but over longer periods very slow reversion was detectable.

Fig. 3. Time course of spectral changes on addition of propionyl-CoA to SCAD

Propionyl-CoA was added to SCAD to give final concentrations of 93 μM -SCAD (by flavin) and 466 μM -propionyl-CoA. Spectra (all corrected for dilution) were recorded at the time intervals indicated: 1, before propionyl-CoA addition, and 2, 95 s, 3, 335 s, and 4, 815 s after propionyl-CoA addition.

Table 1. Relationship between the extent of flavin reduction and charge-transfer band formation on aerobic addition of various straight-chain acyl-CoA compounds to SCAD, and the value of k_{cat} in catalytic assays

k_{cat} values are from Shaw & Engel (1984). Percentage flavin reduction was calculated from A_{448} before and after addition of a 4-fold excess of substrate over flavin. Charge-transfer band magnitude was taken as the ratio A_{560}/A_{448} , with A_{560} being measured after substrate addition and A_{448} being the initial value before substrate addition.

Acyl-CoA	k_{cat} (s^{-1})	Flavin reduction (%)	Magnitude of charge- transfer band
Butyryl-CoA	2.5	73	0.230
Pentanoyl-CoA	3.3	71	0.185
Hexanoyl-CoA	1.35	45	0.105
Heptanoyl-CoA	0.25	14	0.048
Octanoyl-CoA	0.1	5	0.032

Reversion was more thoroughly tested by using an alternative high-affinity ligand, acetoacetyl-CoA. A sample of inactivated enzyme was dialysed overnight to remove any excess of propionyl-CoA. The spectrum indicated partial (about 15%) recovery of A_{448} during dialysis (Fig. 4). Addition of acetoacetyl-CoA then progressively converted the spectrum into that of a normal and fully formed enzyme-acetoacetyl-CoA complex with characteristic long-wavelength absorbance centred at 555 nm (Shaw & Engel, 1984) (Fig. 4). This shows that the effects of propionyl-CoA are reversible.

Reversibility was also indicated by the significant acceleration of 2,6-dichlorophenol-indophenol reduction seen when assays of inactivated enzyme, with butyryl-CoA as the substrate, were monitored for several minutes.

The absorbance changes at 448 nm during inactivation exhibited pseudo-first-order kinetics. By using the final A_{448} as an equilibrium value, apparent first-order rate constants ($k_{app.}$) were determined for various propionyl-CoA concentrations from the slopes of plots of $\ln[A_0 - A_e]/(A_t - A_e)$ against time, where A_0 was the initial A_{448} , A_e the final A_{448} and A_t the A_{448} at time t . The dependence of $k_{app.}$ on propionyl-CoA concentration showed a saturation effect, giving a

linear double-reciprocal plot (Fig. 5). The results indicate a saturable initial encounter with SCAD followed by the first-order formation of the bleached complex. The $k_{app.}$ values for these spectral changes with 200 μM - and 500 μM -propionyl-CoA were 5.8×10^{-3} and $7.2 \times 10^{-3} \text{ s}^{-1}$ respectively. Since these values are almost identical with the $k_{app.}$ values for inactivation at the same propionyl-CoA concentrations, bleaching and inactivation are clearly related events.

Nature of the flavin released from propionyl-CoA-treated SCAD

The first-order decrease in SCAD activity caused by such a seemingly innocuous compound as propionyl-CoA suggests that a form of suicide inhibition is occurring (Rando, 1974). Like many previously documented suicide inhibitors of flavoproteins, propionyl-CoA may be forming a flavin adduct (Maycock, 1980; Walsh *et al.*, 1978; Abeles & Maycock, 1976). This is borne out by the decrease in A_{448} and the appearance of an absorption band at 335 nm, both changes indicative of the formation of an *N*-5- or *N*-5,*C*-4(*a*)-alkylated reduced flavin (Hemmerich *et al.*, 1971; Ghisla *et al.*, 1974).

The flavin from propionyl-CoA-modified SCAD was released in separate experiments with

Fig. 4. Time course of spectral changes on addition of acetoacetyl-CoA to propionyl-CoA-modified enzyme. Final concentrations of 6.4 μM -SCAD (by flavin) and 44 μM -propionyl-CoA were incubated at room temperature for 20 min. After overnight dialysis (during which about 15% re-oxidation occurred) acetoacetyl-CoA was added to a final concentration of 12.8 μM . Spectra were recorded at the time intervals indicated: 1, before acetoacetyl-CoA addition, and 2, 50 min, and 3, 4 h after acetoacetyl-CoA addition.

Fig. 5. Double-reciprocal plot of pseudo-first-order rate constants for the decrease in A_{448} against propionyl-CoA concentration

SCAD at a final concentration of 6.7 μM (by flavin) was incubated at 25°C with propionyl-CoA at various concentrations. A_{448} was monitored continuously. First-order rate constants were determined as described in the text.

5M-guanidinium chloride, 5% (w/v) sodium dodecyl sulphate, and 5% (w/v) trichloroacetic acid with ether extraction. The three procedures yielded modified flavins with almost identical absorption spectra, showing a long-wavelength band centred at 600 nm and maxima at 465 and 350 nm (e.g. Fig. 6). By contrast, unmodified FAD was released with trichloroacetic acid from a control sample of enzyme treated with butyryl-CoA.

The spectrum in Fig. 6 was stable for at least 2 h and is reminiscent of a neutral flavin semiquinone (Beinert & Sands, 1961). When this species was subjected to e.p.r. spectroscopy a radical signal with a g -value of 2.0037 and hyperfine splitting was indeed detected (George *et al.*, 1984). Significantly, the intact propionyl-CoA-modified enzyme gave no e.p.r. signal, indicating that the free radical is only produced on release of flavin from the enzyme. Integration of the e.p.r. signal (recorded at liquid-N₂ temperature) of the flavin extracted from propionyl-CoA-treated enzyme (71.6 μ M total flavin) with trichloroacetic acid gave a concentration of 44 μ M free radical (George *et al.*, 1984). Since the change in A_{448} indicates that only 70% of the flavin was initially modified, this represents 0.88 mol of free radical/mol of modified flavin, implying almost quantitative conversion of the modified flavin into free radical.

Although most flavin free radicals are unstable in air, a few air-stable radicals have been generated by the re-oxidation of reduced *N*-5-alkyl-lumi-flavins (Hemmerich *et al.*, 1967; Walker *et al.*, 1970; Kemal *et al.*, 1977). By analogy, it seems likely that a reduced *N*-5-alkylflavin is generated in the SCAD active site, and that release from the protein exposes it to O₂, so generating a flavin semiquinone. In agreement with this hypothesis, anaerobic

release of the modified flavin with 5M-guanidinium chloride yielded no long-wavelength absorption until air was re-admitted.

Fate of the CoA moiety on addition of propionyl-CoA to SCAD

A concentrated solution of the flavin extracted from SCAD treated with propionyl-[G-³H]CoA was injected on to an analytical C₁₈ reverse-phase h.p.l.c. column and eluted with a gradient of methanol in 10 mM-KH₂PO₄. Fractions, collected manually, were analysed by scintillation counting and absorption spectroscopy (Fig. 7). Peak 1 contained negligible radioactivity and had a featureless absorption spectrum. Peak 2 possessed a u.v.-absorption spectrum identical with that of propionyl-CoA, and contained the amount of radioactivity expected on the basis of A_{260} . This peak may result from incomplete dialysis of excess propionyl-CoA or from the reversible nature of adduct formation. Peak 3 had the spectral characteristics of FAD, although some carry-over of material from peak 2 was indicated by the presence of a little radioactivity. Peak 4 contained significant radioactivity and its absorption spectrum (Fig. 8) appears to be that of a flavin radical (Walker *et al.*, 1970). The quantification of [G-³H]CoA under peak 4 gave 17.4 nmol. Calculation from A_{260} gave 15.8 nmol, based on an estimated ϵ_{260} of 53800 M⁻¹·cm⁻¹ (the sum of ϵ_{260} values for FAD and CoA). Thus the modified flavin contains CoA. The free FAD in peak 3 constituted 31% of the total eluted flavin (peak 3 plus peak 4). This roughly matches the 22% of unmodified flavin expected on the basis of residual activity, although the difference may reflect some reversion of the adduct to FAD during sample preparation.

Fig. 6. Absorption spectrum of flavin released with trichloroacetic acid from propionyl-CoA-modified SCAD. Final concentrations of 45 μ M-SCAD and 223 μ M-propionyl-CoA were incubated at room temperature for 20 min. Then 0.5 vol. of 15% trichloroacetic acid was added, denatured protein was removed by centrifugation, and the supernatant was extracted five times with ether and its absorption spectrum measured.

Re-activation of propionyl-CoA-modified enzyme

The results so far suggest that propionyl-CoA partially inhibits ox liver SCAD by the formation of an adduct with the bound flavin cofactor. This adduct appears to be in equilibrium with the active, unmodified, cofactor. The reverse reaction of this equilibrium was investigated more directly by exposing propionyl-CoA-modified enzyme to high concentrations of butyryl-CoA. This high-affinity substrate was intended to 'trap' any free active enzyme that might result from the equilibrium.

In two experiments, 3.0 μ M- and 33 μ M-SCAD (by flavin) were inactivated to equilibrium by a 30 min incubation at 25°C with a 10-fold molar excess of propionyl-CoA. These inactivated enzyme preparations were diluted 10-fold into final concentrations of 200 μ M- and 1 mM-butryl-CoA respectively. Samples were withdrawn at timed

Fig. 7. H.p.l.c. chromatogram of material extracted with trichloroacetic acid from propionyl-[G-³H]CoA-treated SCAD

SCAD (164 nmol, by flavin) was incubated with a 5-fold excess of propionyl-[G-³H]CoA for 30 min at room temperature, and dialysed against 4 mM-potassium phosphate buffer, pH 7.6, to remove as much propionyl-CoA as possible, without allowing too much re-oxidation, and to decrease the salt content. Flavin was extracted with 5% trichloroacetic acid, and, after precipitation of denatured protein, the supernatant was extracted five times with ether, freeze-dried and resuspended in 250 μ l of cold water. A 100 μ l portion of concentrated extract was injected on to an analytical C₁₈ reverse-phase h.p.l.c. column and eluted with a 3.5–50% gradient (----) of methanol in 10 mM-KH₂PO₄ at 0.9 ml/min. The absorbance of the column eluate was monitored at 254 nm (—) in a flow cell. [G-³H]CoA in each eluate peak was determined by liquid-scintillation counting (histogram).

intervals and their initial catalytic activity towards butyryl-CoA was measured in the standard dye-reduction assay. In both cases a slow increase in SCAD activity was observed, finally resulting in almost complete re-activation. Plots of $\ln[(A_0 - A_t)/(A_i - A_t)]$ (where A_0 is activity before re-activation, A_t is activity at time t and A_i is final activity) versus time were linear for both experiments, and the first-order rate constants deduced from the gradients were $1.65 \times 10^{-3} \text{ s}^{-1}$ for the first experiment (3 μ M-SCAD) and $2 \times 10^{-3} \text{ s}^{-1}$ for the second (33 μ M-SCAD).

Fig. 8. Absorption spectrum of the FAD-propionyl-CoA adduct purified by h.p.l.c.

Peak 4 from the h.p.l.c. chromatogram illustrated in Fig. 7 was manually collected and its absorption spectrum was measured.

Discussion

The results show that, although propionyl-CoA can act as a normal substrate for SCAD in the catalytic assay, its turnover is very slow (about 1.2% of that for butyryl-CoA) and is accompanied by a gradual inactivation of the enzyme by the reversible formation of a CoA-containing, and catalytically incompetent, adduct with the bound flavin cofactor.

In the absence of efficient electron acceptors, the modification reaction proceeds without competition from the normal catalytic reaction. If the enzyme is saturated with propionyl-CoA, the inactivation reaches an equilibrium in which the proportions of active (unmodified) and inactive (modified) enzyme are 22% and 78% respectively. This we take to represent the equilibrium, in Scheme 1, between the enzyme-propionyl-CoA Michaelis complex and the covalently modified enzyme. The equilibrium constant for this reaction can therefore be calculated to be 3.54.

The apparent first-order rate constant for approach to equilibrium in a reversible reaction is equal to the sum of the two true opposing rate constants, in this case k_{+2} and k_{-2} in Scheme 1 (Frost & Pearson, 1961). The extrapolated value for this apparent constant from Fig. 5 is $8.9 \times 10^{-3} \text{ s}^{-1}$. Since the equilibrium constant gives the ratio of k_{+2} and k_{-2} , the individual values of k_{+2} and k_{-2} can be calculated to be $6.9 \times 10^{-3} \text{ s}^{-1}$ and $2.0 \times 10^{-3} \text{ s}^{-1}$ respectively. A direct estimate of k_{-2} was obtained by kinetic analysis of the re-activation of the enzyme on dilution and incuba-

Scheme 1.

Abbreviation: PES, *N*-ethylphenazinium ethosulphate.

tion with a very large molar excess of butyryl-CoA. Under these conditions the enzyme can be expected to be re-activated at a rate limited by k_{-2} . The values obtained are in good agreement with that predicted above, thus corroborating the mechanism illustrated in Scheme 1.

k_{cat} for the catalytic reaction with propionyl-CoA was 0.03 s^{-1} . The partitioning of the Michaelis complex between catalysis and inactivation (i.e. k_{cat}/k_{+2}) thus appears to be approximately in the ratio 4.3:1. However, there are some indications that this analysis may represent an oversimplification. It is puzzling that concentrations of propionyl-CoA giving identical levels of inactivation (78%) nevertheless give different inactivation rate constants (Fig. 1). It is possible that further investigation may require the inclusion of additional intermediates. These studies clearly show, however, that propionyl-CoA is a pseudo-suicide substrate, since it fulfils the usual criteria for a suicide substrate (Maycock, 1980) except that inhibition is reversible.

Several suicide substrates have been employed previously in attempts to elucidate the mechanism of catalysis by acyl-CoA dehydrogenases, and the results point to the initial formation of a substrate carbanion (Ghisla *et al.*, 1980; Frerman *et al.*, 1980; Wenz *et al.*, 1981). Although little is known about subsequent electron transfer to the flavin, experiments with lactate oxidase (Massey *et al.*, 1980; Ghisla & Massey, 1980) and D-amino acid oxidase (Porter *et al.*, 1973) have suggested that in the catalytic cycle of these flavoenzymes the activated substrate carbanion may form a covalent intermediate with the flavin cofactor.

The present investigation demonstrates that, in contrast with the adduct formed in the glycollate reaction of lactate oxidase, the adduct formed by propionyl-CoA and SCAD-bound flavin is not a catalytic intermediate. Rather it represents a 'derailment' of the normal catalytic process. Since other acyl-CoA dehydrogenase suicide substrates tend to possess unusual chemical groupings adjacent to the reactive bond, the chemical similarity

of propionyl-CoA to a normal substrate (e.g. butyryl-CoA) makes the observed inactivation particularly interesting. In accordance with Maycock's (1980) suggestion, the elucidation of the structure of this flavin-substrate adduct may provide some insight into the mechanism of ox liver SCAD.

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