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Acyl-Coenzyme A Dehydrogenase from Pig Kidney. Purification and Properties†

Colin Thorpe,* Rowena G. Matthews, and Charles H. Williams, Jr.

ABSTRACT: Comparatively large amounts of an acyl-CoA dehydrogenase have been obtained from pig kidney by a procedure which does not involve prior isolation of mitochondria. The pattern of substrate specificity and the extent of substrate-induced bleaching of the flavin chromophore, using butyryl-, octanoyl-, and palmitoyl-CoA, suggest that the enzyme be classified as a general acyl-CoA dehydrogenase. The purified flavoprotein exhibits absorbance ratios at 272, 373, and 446 nm of 5.7:0.65:1.0, respectively, with an extinction coefficient for bound flavin adenine dinucleotide (FAD) of 15.4 mM^{-1} cm^{-1} at 446 nm. Gel filtration, $NaDodSO_4$ gel electrophoresis, and amino acid analysis indicate that the enzyme is a tetramer, comprised of subunits of about 42 000 molecular weight, containing 3–4 molecules of FAD as isolated. The blue, neutral flavosemiquinone is formed during anaerobic titration of the enzyme with dithionite (observed ϵ_{560} = 2.8 mM^{-1} cm^{-1}). A similar level of semiquinone is formed during deazaflavin/light reduction, but no long-wavelength intermediates are observed on reduction with borohydride. Full reduction of the flavin requires 1.2 mol of dithionite. Back titration of the fully reduced enzyme with ferricyanide yields considerable levels of semiquinone (observed ϵ_{560} = 4.1 mM^{-1} cm^{-1}) and suggests that the extinction coefficient of this species is approximately 5.9 mM^{-1} cm^{-1}. The disproportionation equilibrium between semiquinone, oxidized, and fully reduced flavin forms is attained very slowly in the absence of mediators.

Mammalian fatty acyl-CoA dehydrogenases are flavoproteins participating in the first dehydrogenation step of fatty acid β-oxidation which leads to the formation of α,β-unsaturated acyl-CoA derivatives (Beinert, 1963). The reducing equivalents are then passed to a second component, electron transfer flavoprotein (ETF;[1] Crane et al., 1956), which interacts with the respiratory chain, possibly at the level of a recently identified iron–sulfur flavoprotein (Ruzicka & Beinert, 1977). Three categories of acyl-CoA dehydrogenases have been described with overlapping substrate specificity patterns. Short-chain or butyryl-CoA dehydrogenase acts on C_4–C_6 acyl-CoA (Green et al., 1954), general or acyl-CoA dehydrogenase acts on C_4–C_{16} acyl-CoA with peak activity toward C_{10} acyl-CoA (Crane et al., 1956), and long-chain or palmitoyl-CoA dehydrogenase acts on C_6–C_{16} acyl-CoA with maximal activity toward C_{12} acyl-CoA (Hauge et al., 1956). These three dehydrogenases are thought to be closely associated in vivo (Crane et al., 1956; Stanley & Tubbs, 1975). Despite the widespread belief that β-oxidation in mammals occurs exclusively intramitochondrially, recent evidence suggests that long-chain fatty acids are also oxidized by a

† From the Veterans Administration Hospital and the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48105. *Received July 27, 1978.* This work was supported in part by Grant GM-21444 from the National Institute of General Medical Sciences, National Institutes of Health, U.S. Public Health Service (C.H.W.), and by Michigan Memorial Phoenix Project 534 (R.G.M.). The work was aided in part by U.S. Public Health Service Grant AM 12734 (equipment grant) to the Department of Biological Chemistry, University of Michigan. A preliminary account of some of this work was presented at the 6th International Symposium on Flavins and Flavoproteins, March 1978, Kobe, Japan.

* Present address: Department of Chemistry, University of Delaware, Newark, DE 19711.

[1] Abbreviations used: ETF, electron transferring flavoprotein; DCI, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; PMSF, phenylmethanesulfonyl fluoride; FAD, flavin adenine dinucleotide; M_r, relative molecular mass (molecular weight).

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similar pathway in rat liver peroxisomes (Lazarow & de Duve, 1976; Lazarow, 1978). The enzymes responsible for the initial desaturation step have not been characterized from this source.

Previously published isolation schemes for mammalian acyl-CoA dehydrogenases use liver or heart as source tissue and yield comparatively small amounts of purified enzyme. The low yields associated with these preparations have seriously restricted study of these metabolically important flavoproteins. We have observed that pig kidney is a rich source for an acyl-CoA dehydrogenase of general specificity. This paper describes the purification and properties of this enzyme.

Experimental Procedures

Materials. CoA derivatives were obtained from P-L Biochemicals. PMS, PMSF, DCI, and FAD (further purified by the method of Massey & Swoboda, 1963) were from Sigma. 3,10-Dimethyl-5-deazaisoalloxazine was a gift from Dr. V. Massey. Sephacryl S-200 and AH-Sepharose 4B were obtained from Pharmacia.

Methods. Concentrations of pig kidney acyl-CoA dehydrogenase are expressed with respect to enzyme-bound flavin using an ϵ_{446} value of 15.4 mM^{-1} cm^{-1} (see later).

Visible and ultraviolet spectra were recorded on a Cary 118C spectrophotometer interfaced to a minicomputer (Williams et al., 1979). Assays were conducted using a Beckman DU or a Cary 118C spectrophotometer. Gels were examined using a Gilford linear transport 2410-S gel scanning attachment using 0.2-mm aperture plates.

Release of flavin by the trichloroacetic acid extraction method was performed essentially as described by Mayhew & Massey (1969). The fluorescence of diluted portions of this extract was examined in a Turner Model 111 fluorimeter using a Bausch and Lomb 444 exciting filter and Kodak-Wratten 2A/12 emitting filter.

Assays. Since pig kidney ETF was unavailable, and because of problems which have been previously encountered in the use of ETF to assay acyl-CoA dehydrogenases (Hall & Kamin, 1975; Hall et al., 1976), we have substituted PMS as a mediator (Hauge, 1956; Engel & Massey, 1971a; Singer, 1975). The assay mixture used to monitor purification was 33 μM octanoyl-CoA (or, in some cases, butyryl- or palmitoyl-CoA), 30 μM DCI, and 1.4 mM PMS in 0.7 mL of 20 mM phosphate buffer, pH 7.6, containing 60 μM EDTA at 25 °C. Enough enzyme was added to give a decrease in DCI absorbance at 600 nm (ϵ = 21 mM^{-1} cm^{-1}; Steyn-Parvé & Beinert, 1958) of approximately 0.06/min, after subtraction of a nonenzymatic background rate of about 0.004/min. The enzymatic reduction of DCI in the absence of 1.4 mM PMS is insignificant. The observed turnover number per enzyme flavin under these conditions is about 190 min^{-1} using octanoyl-CoA. Hall & Kamin (1975), using the physiological mediator ETF, report an extrapolated turnover number of 500 min^{-1} per enzyme flavin at infinite ETF concentration.

$NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis. These runs were kindly performed by S. Krezoski, in the laboratory of Dr. M. J. Coon, University of Michigan, by the procedure described by Haugen & Coon (1976) using a discontinuous buffer system according to Laemmli (1970). Approximately 0.3 μg of standard proteins (chymotrypsinogen A, lactate dehydrogenase, aldolase, ovalbumin, L-glutamate dehydrogenase, catalase, bovine serum albumin, and phosphorylase *a*) and 1 μg of acyl-CoA dehydrogenase were applied to the gel. The proteins were stained with Coomassie blue.

Activity Stain. Gels were stained for activity in 45 mM phosphate buffer, pH 7.2, containing 33 μM octanoyl-CoA, 380 μM PMS, and 60 μM iodonitrotetrazolium. After several hours of incubation in the dark at room temperature, a red band appears coincident with the enzyme. The gels were washed and stored in 7% acetic acid.

Table I: Purification of an Acyl-CoA Dehydrogenas from Pig Kidney

step	total A_{280}	total A_{450}[a]	A_{280}/A_{450}	total units[b]
(1) supernatant (47 g of protein[c]) from homogenate of 1070 g of pig kidney; after pH 5.8 precipitation				459
(2) DE-52 batch adsorption of neutralized supernatant: column elution of yellow fractions (4.4 g of protein)	3553	127	28	391
(3) calcium phosphate gel-cellulose column	684	44	15.6	
(4) 40–80% ammonium sulfate precipitation	383	36	10.7	
(5) DE-52 column				
(a) pale green fractions	14	0.9	16	1.4
(b) yellow fractions	109	15.9	6.9	171
(c) side fractions from (b)	101	8.9	11.4	79
(6) Sephacryl S-200, gel filtration of material from (5b)	54	9.6	5.6	114
(7) AH-Sepharose 4B column (from 6)	39	7.4	5.25	92

[a] This includes the contribution of other chromophores, e.g., hemoproteins and iron-sulfur centers. [b] Micromoles of DCI reduced per min under standard assay conditions using octanoyl-CoA (see Methods). [c] Determined by the biuret reaction.

Gel Filtration. Estimation of native molecular weight was performed on a 89 × 1.4 cm Sephacryl S-200 column equilibrated with 100 mM phosphate, pH 7.6, at 4 °C and eluted at 13 mL/h. Standard proteins were ribonuclease, chymotrypsinogen A, ovalbumin, bovine serum albumin, pig heart lipoamide dehydrogenase, and human γ-globulin.

Amino Acid Analysis. Amino acid analyses were performed on a Beckman 120B analyzer modified for single-column operation and interfaced to a PDP 8/E minicomputer, which controls operation and data acquisition. Samples were hydrolyzed at 110 °C in constant boiling HCl (Moore & Stein, 1960). Tryptophan analyses were performed, after hydrolysis of the protein in methanesulfonic acid (Liu & Chang, 1971), using Beckman AA-15 resin essentially according to the manufacturer's procedure.

Enzyme Purification. Unless otherwise stated, all operations were performed at 0–4 °C and all buffers contained 0.3 mM EDTA. Ammonium sulfate saturation levels refer to 25 °C. A summary of the purification scheme is shown in Table I. Pig kidneys were stored at −70 °C. Cortical tissue was cut from 1070 g of partially thawed kidneys. The pieces (780 g) were combined with 2 L of 50 mM phosphate buffer, pH 5.9, containing 0.35 g of PMSF freshly added in 8 mL of 2-propanol. The mixture was blended for 2.5 min at top speed in a Waring blender and then filtered through two layers of cheesecloth. The filtrate was adjusted to pH 5.8 with 4 M acetic acid and centrifuged at 25000*g* for 30 min. The supernatant (1800 mL) was adjusted to pH 7 with 1 M ammonium hydroxide, and 360 mL of packed wet Whatman DE-52, previously equilibrated in 50 mM phosphate, pH 7.2, was added. The slurry was gently stirred for 90 min and then filtered through Whatman No. 4 paper on a large Büchner funnel. The DE-52 was washed with 3 × 1 L of 50 mM phosphate buffer, pH 7.2, suspended in 300 mL of the same buffer, and poured into a 2.5-cm diameter column. The yellow-brown packed gel was washed at 48 mL/h with 300 mL of the same buffer; a pale brown solution of yellow-green

fluorescence was eluted. On changing to 0.3 M phosphate buffer, pH 7.2, acyl-CoA dehydrogenase was eluted as a bright yellow band which became more distinct as the solvent front moved down the column. The yellow fractions were pooled (209 mL) and taken to 85% ammonium sulfate. The precipitate was redissolved in approximately 100 mL of 0.1 M phosphate buffer, pH 7.6, and dialyzed overnight against 2 L of this buffer, containing 70 mg of PMSF added in 1.6 mL of 2-propanol. The solution was then applied to a 2.5 × 40 cm calcium phosphate gel–cellulose column (Massey, 1958). The adsorbed protein was washed with 70 mL of 0.1 M phosphate buffer, pH 7.6, and eluted at 27 mL/h with 0.2 M phosphate buffer, pH 7.6. (We experience variability in the ability of calcium phosphate gel, prepared by the method of Swingle & Tiselius (1951), to bind flavoproteins. The preparation used for this column was worse than average; when another batch of gel was used, elution of the acyl-CoA dehydrogenase required 0.4 M phosphate buffer, pH 7.6.) Most of the heme-containing contaminants elute before the flavoprotein fractions. The combined yellow fractions (118 mL) were fractionated with ammonium sulfate, and the 40–80% precipitate was redissolved in 4 mL of 100 mM phosphate buffer, pH 7.6, and dialyzed against 1 L of 50 mM phosphate buffer, pH 7.2. The solution was applied to a DE-52 column (2.5 × 44 cm) equilibrated with this buffer and the dehydrogenase adsorbed as a bright yellow band. The column was developed at 58 mL/h using a linear gradient formed by mixing 600 mL each of 50 and 250 mM phosphate buffer, pH 7.2. Small amounts of a pale green material were followed by a broad yellow band peaking after 720 mL of the gradient. Fractions were pooled according to their 280/450 nm absorbance ratios (see Table I), precipitated with 80% ammonium sulfate, and dialyzed against 100 mM phosphate buffer, pH 7.6. Enzyme was stored at −20 °C in this buffer.

At this stage, it was found convenient to further purify portions of the dehydrogenase (280/450 ratio = 6.9, see Table I) as needed, keeping the remainder frozen. The enzyme (1 mL, 167 nmol) was gel filtered on a Pharmacia Sephacryl S-200 column (89 × 1.5 cm) equilibrated with 20 mM phosphate buffer, pH 7.6. Tubes were combined according to their 280/450 nm ratio and 6 mL of the best fractions (100 nmol, ratio 5.6) were applied to an AH-Sepharose-4B column (25 × 0.6 cm). The enzyme sticks tightly as a narrow band in this buffer but may be eluted using 0.16 M phosphate, pH 7.6. Fractions were pooled, yielding 77 nmol of acyl-CoA dehydrogenase (ratio 5.25), together with side fractions of lower purity. Side fractions from steps 5 to 7 may be recycled by a combination of calcium phosphate gel–cellulose, gel filtration, and AH-Sepharose-4B steps described above.

Results and Discussion

Previous purification methods for mammalian acyl-CoA dehydrogenases involved the isolation of mitochondria, followed by their disruption during the preparation of acetone powders (Crane et al., 1956) or by sonication (Hall & Kamin, 1975). In the scheme outlined in Table I, pig kidney cortex is homogenized into hypotonic buffer containing the protease inhibitor PMSF, and the acyl-CoA dehydrogenase is adsorbed from the supernatant by DE-52 batch treatment. The remaining supernatant exhibits a strong yellow-green fluorescence, but no detectable acyl-CoA dehydrogenase activity (see Methods). If kidney ETF is solubilized by our homogenization step, it would not be expected to be bound to DE-52 under these conditions and could therefore contribute to the fluorescence of the supernatant fraction (Hall & Kamin, 1975; Beinert & Lee, 1963). Purification of ETF from kidney has

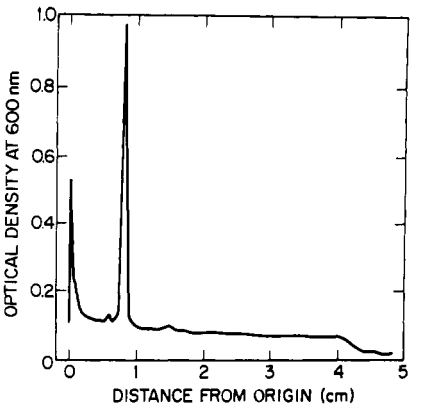


FIGURE 1: Disc gel electrophoresis of pig kidney general acyl-CoA dehydrogenase. Electrophoresis of 4 μg of dehydrogenase was performed in 7.5% gels by the method of Davis (1964). Gels were stained with Amido Black.

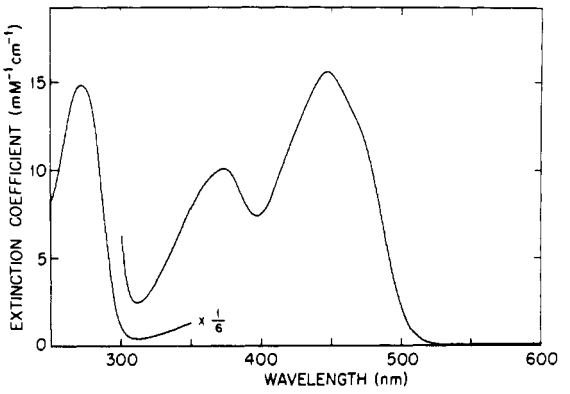


FIGURE 2: Visible and ultraviolet spectrum of pig kidney general acyl-CoA dehydrogenase. Spectra were recorded in 160 mM phosphate, pH 7.6, at 25 °C.

not been attempted in this work. Fractions eluting from the second DE-52 step (fractions 5b,c; Table I) were assayed with butyryl-, octanoyl-, and palmitoyl-CoA substrates (see Methods). A constant ratio of activities (0.1, 1.0, and 0.2, respectively) was maintained across the yellow peak. Since the short-chain, general, and long-chain mammalian acyl-CoA dehydrogenases differ in their substrate specificity (Beinert, 1963) and can be partially separated by DE-52 chromatography (Hall & Kamin, 1975), this result suggests that predominantly one class of dehydrogenase of general specificity is present in fractions 5b,c. This conclusion is supported by disc gel electrophoresis and substrate bleaching patterns (see later). Fraction 5a represents a small amount of pale green material (λ_{max} 440 nm) with a low intensity, long wavelength tail extending to 800 nm. This fraction exhibits low activities toward butyryl-, octanoyl-, and palmitoyl-CoA in the ratio of 1:1:1. Butyryl-CoA dehydrogenases have been isolated in green forms from a variety of sources (Beinert, 1963; Engel & Massey, 1971a), and it is possible that a short-chain acyl-CoA dehydrogenase is responsible for the green color of fraction 5a. Fraction 5b is further purified in two steps to yield approximately 470 nmol of enzyme-bound flavin from 1 kg of pig kidney. For comparison, Hall & Kamin (1975) obtain about 30 nmol of enzyme flavin/kg of pig liver. Enzyme from step 7 shows a sharp band on disc gel electrophoresis with two very minor contaminants (Figure 1). At higher protein levels, the band can be identified by its yellow color or by using an activity stain (see Methods).

The visible and ultraviolet absorption spectrum of the kidney enzyme is shown in Figure 2. The visible spectrum is similar

Table II: Properties of Mammalian Acyl-CoA Dehydrogenases[a]

	pig liver general acyl-CoA dehydrogenase		beef heart long-chain acyl-CoA dehydrogenase, Hall et al. (1976)	pig kidney general acyl-CoA dehydrogenase, this work
	Crane et al. (1956)	Hall & Kamin (1975)		
absorbance ratios	275/370/447 6.7:0.74:1.0	270/370/445 8.0:0.6:1.0	275/360/448 6.3:0.74:1.0	272/280/373/447 5.7:5.3:0.65:1.0
min M_r/FAD	91 000	40 500[b]	38 400[b]	47 700
subunit M_r	nd[d]	42 000	38 500	42 000
native M_r	140 000–200 000	nd	nd	160 000
substrate bleaching (%); C_4:C_8:C_{16} acyl-CoA	55:72:35 (300 μM)[c]	62:82:15 (100 μM)	4:70:61 (50 μM)	31:61:26 (13 μM) 70:79:37 (100 μM)

[a] Taken from the indicated references. [b] Estimated using an assumed extinction coefficient for bound FAD; see text. [c] The concentration of acyl-CoA derivatives used in the bleaching experiments. [d] nd = not determined.

to that of other acyl-CoA dehydrogenases of general or long-chain specificity (Crane et al., 1956; Hall & Kamin, 1975; Hall et al., 1976), although the near-UV trough region and the ratio of UV/visible peak absorbance are significantly lower than previously reported (Table II). The extinction coefficient of the bound flavin chromophore at 446 nm (15.4 mM^{-1} cm^{-1}) is unusually high, although, for example, values of 14.1 and 14.6 mM^{-1} cm^{-1} have been reported for glucose oxidase (Swoboda & Massey, 1965) and pyruvate oxidase (Williams & Hager, 1966), respectively. This extinction coefficient was determined in two ways. First, the flavin in an enzyme solution of known absorbance was released by 4.5 M guanidine hydrochloride in 100 mM phosphate buffer, pH 7.6, and the resulting absorbance was compared with that of a known concentration of FAD under the same conditions. Guanidine hydrochloride, at 4.5 M, causes a 4% increase in ϵ_{450} over that in phosphate buffer alone (Arscott & Williams, unpublished results). The visible spectrum of the flavin released from the enzyme was identical with that of authentic FAD. Flavin was also liberated and quantitated by the trichloroacetic acid method (Mayhew & Massey, 1969). Incubation of a diluted sample of the neutralized trichloroacetic acid supernatant with *Naja naja* venom resulted in a 10-fold increase in fluorescence emission, as expected for FAD (Bessey et al., 1949). The average of eight determinations of the extinction coefficient was 15.4 ± 0.3 mM^{-1} cm^{-1}.

The approximate molecular weight of the native protein was estimated to be 160 000 using a calibrated gel filtration column (see Methods). A single band of approximately 42 000 molecular weight is observed on $NaDodSO_4$ gels in the presence of 2-mercaptoethanol (see Methods), in good agreement with the subunit molecular weight determined for the pig liver enzyme (Table II). These observations support the proposed tetrameric structure for mammalian acyl-CoA dehydrogenases (Hall & Kamin, 1975; Hall et al., 1976).

The amino acid composition of the kidney enzyme is shown in Table III. The minimum molecular weight per FAD of this preparation is 47 700, some 10% higher than the estimated subunit molecular weight. A possible explanation for this discrepancy is that a corresponding slight loss of flavin from the tetramer has occurred during enzyme purification. Earlier observations suggest that the pig liver general acyl-CoA dehydrogenase is prone to loss of FAD, with a concomitant increase in the 275/447 nm absorbance ratios. Thus, two samples of the enzyme having approximate minimum molecular weights per FAD of 175 000 and 91 000 (Table II) and absorbance ratios of 12.9 and 6.7, respectively, showed no significant differences in their sedimentation properties in the ultracentrifuge (Crane et al., 1956). More recently, Hall & Kamin (1975) isolated the enzyme by a milder procedure and obtained a preparation with an absorbance ratio of 8.0, which migrated as a single band on disc gel electrophoresis. In calculating a minimum molecular weight per FAD of 40 500, they assumed an extinction coefficient of 11.3 mM^{-1} cm^{-1} for enzyme-bound flavin. If the value of 15.4 mM^{-1} cm^{-1}, obtained in this work, also applies to the pig liver enzyme, their apparent minimum molecular weight would be raised to 55 000. This again suggests that some loss of flavin has occurred.

Table III: Amino Acid Composition of Pig Kidney General Acyl-CoA Dehydrogenase[a]

amino acid	residues/mol of FAD	residues/ 42 000 M_r
aspartic acid	32.3	28.5
threonine	26.2	23.1
serine	14.9	13.2
glutamic acid	59.5	52.5
proline	21.8	19.3
glycine	42.1	37.1
alanine	48.3	42.6
half-cystine[b]	7.4	6.5
valine	22.2	19.6
methionine	9.3	8.2
isoleucine	27.0	23.8
leucine	30.2	26.6
tyrosine	17.3	15.3
phenylalanine	16.9	14.9
lysine	28.1	24.7
histidine	4.6	4.1
arginine	22.8	20.2
tryptophan[c]	3.7	3.2
taurine[b]	nd[d]	

[a] These values are the mean of duplicate analyses after 24-, 48-, and 72-h hydrolysis. Corrections for decomposition of threonine and serine and for incomplete hydrolysis of valine and isoleucine were made. [b] Values obtained after 48-h hydrolysis in the presence of dimethyl sulfoxide (Spencer & Wold, 1969). [c] Determined after hydrolysis of the protein in methanesulfonic acid (Liu & Chang, 1971; see Methods). [d] nd = not detected.

For the above reasons the amino acid composition shown in Table III is expressed both in terms of the minimum molecular weight per FAD of 47 700 and by using the estimate of 42 000 for the subunit molecular weight. The analysis indicates a low tryptophan content, in common with other flavoprotein dehydrogenases (Williams, 1976), together with relatively few histidine residues. Taurine is included in Table III since it is formed during oxidative hydrolysis of the pantetheine moiety of coenzyme A. The kidney enzyme does not contain significant amounts of bound CoA or CoA derivatives, unlike the butyryl-CoA dehydrogenase of *Megasphaera elsdenii* which, on isolation, contains up to 1 equiv of a tightly bound CoA derivative (Engel & Massey, 1971a).

The cysteine residues in the native enzyme react sluggishly toward 0.5 mM DTNB: after 10 min, 0.1 thiol/FAD had

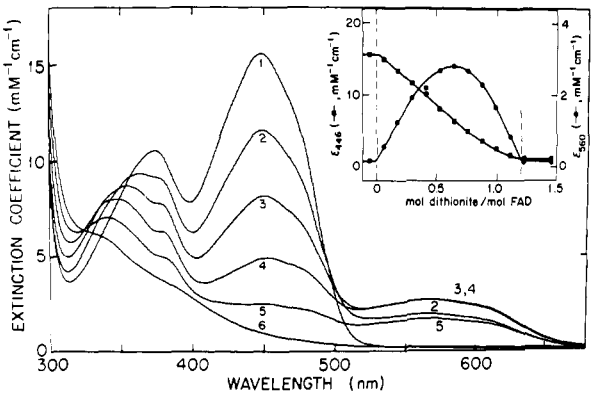


FIGURE 3: Dithionite titration of pig kidney general acyl-CoA dehydrogenase. The protein, 66.5 nmol (enzyme-bound FAD) in 2.4 mL of 100 mM phosphate, pH 7.6, was deoxygenated and titrated with an anaerobic solution of 3.87 mM dithionite at 25 °C (see Methods). Curve 1, 0; 2, 0.29; 3, 0.52; 4, 0.76; 5. 0.99; 6, 1.22 mol of dithionite/mol of FAD. Correction has been made for a small oxygen end point. Intermediate points have been omitted for clarity. The inset shows ϵ_{446} and ϵ_{560} values vs. mol of dithionite/mol of FAD.

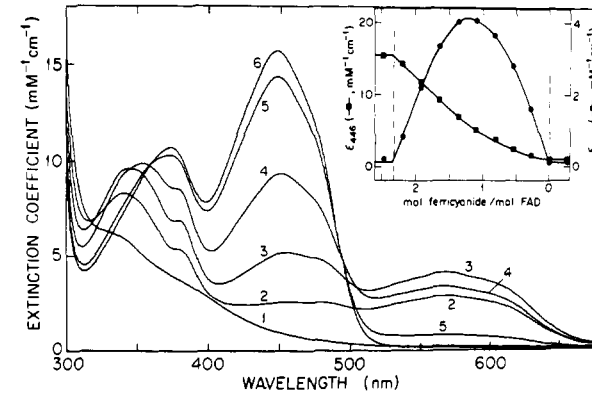


FIGURE 4: Ferricyanide back titration of fully reduced enzyme. Enzyme fully reduced with a slight molar excess of dithionite (from Figure 3) was titrated anaerobically with a solution of 4.57 mM ferricyanide. Curve 1, 0; 2, 0.55; 3, 1.1; 4, 1.65; 5, 2.2; 6, 2.48 mol of ferricyanide/mol of FAD, when corrected for excess dithionite added. Intermediate points have been omitted for clarity.

reacted in phosphate buffer, pH 7.6. In the same buffer containing 4.5 M guanidine hydrochloride, the reaction of a total of 5.6 thiols/FAD required approximately 7 min for completion at 25 °C. No further cysteine residues are exposed at higher perturbant concentrations. Seven half-cystines are indicated by amino acid analysis (Table III).

Figure 3 shows a dithionite titration of acyl-CoA dehydrogenase. In the first phase of the titration (curves 1–3), formation of significant levels of the blue, neutral flavin semiquinone (Massey & Palmer, 1966) is evident from the rise in long-wavelength absorption. Transient formation of this long-wavelength band was observed during reduction of general acyl-CoA dehydrogenase from pig liver by excess dithionite at 4 °C (Beinert & Sands, 1961). In contrast, no semiquinone was detected during dithionite reduction of the yellow form of butyryl-CoA dehydrogenase from *Megasphaera elsdenii* (Engel & Massey, 1971a). Full reduction of the flavin chromophore requires 1.2 mol of dithionite/mol of FAD (inset, Figure 3) and leads to the complete loss of long-wavelength absorbance. The spectral changes seen in Figure 3 were completed rapidly after each addition of dithionite, and no further changes were observed before the next addition of reductant was made (approximately 15 min).

Back titration of the fully reduced enzyme using ferricyanide is shown in Figure 4. Clearly a higher concentration of the blue semiquinone is attained during reoxidation using this obligatory one-electron acceptor than during dithionite reduction (maximal observed ϵ_{560} values are 4.1 and 2.8 mM^{-1} cm^{-1}, respectively). Extrapolation of the extremes of the inset to Figure 4 suggests an ϵ_{560} value of roughly 5.9 mM^{-1} cm^{-1} for fully formed semiquinone under these conditions. Complete reoxidation requires 2.3 mol of ferricyanide/mol of FAD. In the initial phase of the titration, reoxidation by each aliquot of ferricyanide required several minutes for completion. However, the time required increased markedly after maximal semiquinone formation had been reached, extending to several hours between the last few points. In contrast to ferricyanide, very little semiquinone is observed after partial oxidation of fully reduced enzyme by atmospheric oxygen. It is unknown whether this reoxidation proceeds in one-electron steps or directly to yield oxidized flavin and hydrogen peroxide.

The kidney enzyme may be catalytically photoreduced with deazaflavin (Massey & Hemmerich, 1978) using EDTA as the ultimate source of reducing equivalents. Illumination of

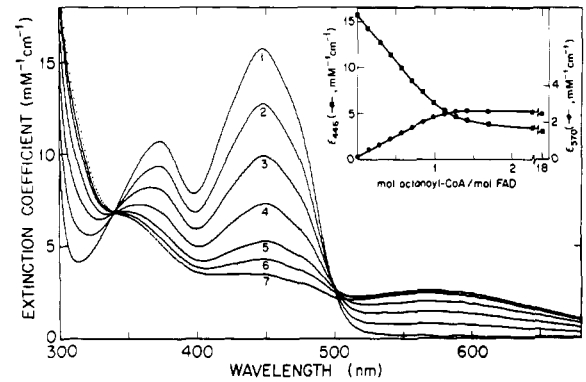


FIGURE 5: Titration of pig kidney general acyl-CoA dehydrogenase with octanoyl-CoA. The enzyme, 25 nmol in 1.86 mL of deoxygenated 100 mM phosphate buffer, pH 7.6, was titrated anaerobically with octanoyl-CoA at 25 °C. Curve 1, 0; 2, 0.28; 3, 0.57; 4, 0.85; 5, 1.13; 6, 1.42; 7, 2.27 mol of substrate/mol of enzyme-bound FAD. Intermediate points have been omitted for clarity (see inset).

18 μM enzyme at 20 °C with 2.3 μM 3,10-dimethyl-5-deazaisoalloxazine and 5 mM EDTA in 100 mM phosphate, pH 7.6, at a distance of 5 cm from a 150-W tungsten bulb, gave maximal blue semiquinone formation after 6 min (observed ϵ_{560} = 2.55 mM^{-1} cm^{-1}). Full reduction required 18 min of light. Insignificant photoreduction of the enzyme occurs over this time period in the absence of deazaflavin.

Engel & Massey (1971a) showed that borohydride reduction of butyryl-CoA dehydrogenase from *Megasphaera elsdenii* leads to formation of the 3,4-dihydroflavin isomer. Anaerobic incubation of the kidney enzyme with a considerable excess of sodium borohydride at pH 7.6 results in slow reduction of the flavin without the formation of spectroscopically significant levels of this isomer. No semiquinone is observed during reduction.

The above results suggest that the disproportionation equilibrium between individual enzyme-bound flavin molecules

$$2EFlH\cdot \rightleftharpoons EFl_{ox} + EFlH_2$$

is attained very slowly in pig kidney general acyl-CoA dehydrogenase in the absence of mediators. Thus, reduction by dithionite, deazaflavin/light, or borohydride or reoxidation by ferricyanide or molecular oxygen proceeds via paths which yield widely varying amounts of flavosemiquinone. This situation is observed with many other flavoproteins (Mayhew & Massey, 1973). A value for the disproportionation equilibrium constant has not yet been obtained.

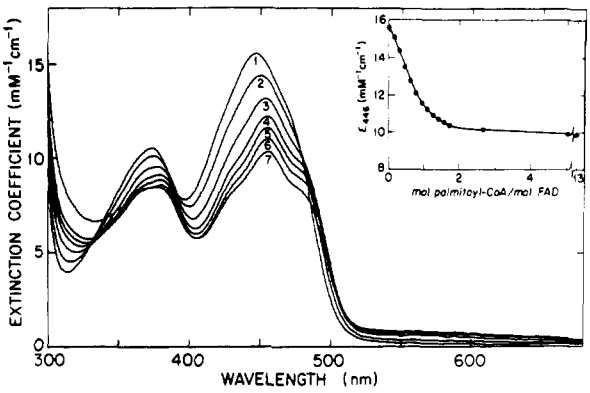


FIGURE 6: Titration of the enzyme with palmitoyl-CoA. The dehydrogenase, 23 nmol in 1.85 mL of deoxygenated 100 mM phosphate buffer, pH 7.6, was titrated anaerobically with palmitoyl-CoA to give the following. Curve 1, 0; 2, 0.31; 3, 0.63; 4, 0.94; 5, 1.26; 6, 1.89; 7, 12.9 mol of substrate/mol of FAD. Intermediate points have been omitted for clarity.

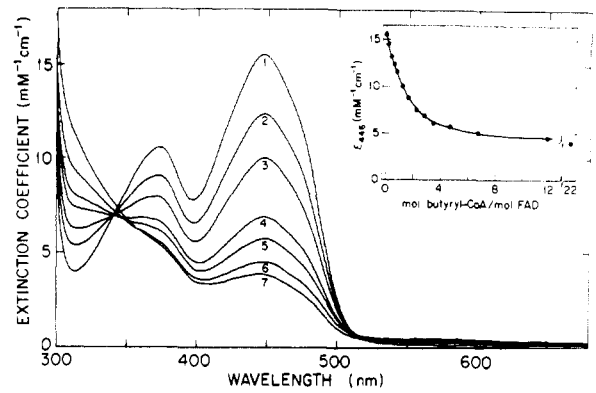


FIGURE 7: Titration of the enzyme with butyryl-CoA. The protein, 23 nmol in 1.84 mL of deoxygenated 100 mM phosphate buffer, pH 7.6, was titrated anaerobically with butyryl-CoA at 25 °C. Curve 1, 0; 2, 0.61; 3, 1.23; 4, 2.86; 5, 4.70; 6, 11.9; 7, 22.1 mol of substrate/mol of FAD. Intermediate points have been omitted for clarity.

Figure 5 shows an anaerobic titration of the pig kidney enzyme with octanoyl-CoA at pH 7.6. Reduction of the flavin chromophore is accompanied by the appearance of a broad long-wavelength band centered around 570 nm (maximal ϵ_{570} = 2.5 mM^{-1} cm^{-1}), with absorbance extending to 800 nm. This band was originally ascribed to a flavosemiquinone (Beinert, 1957). However, in the light of more recent work on bacterial butyryl-CoA dehydrogenase and on other flavoproteins, it probably represents a charge-transfer complex between reduced flavin as the donor and the π system of a bound molecule of the unsaturated thioester as the acceptor (Engel & Massey, 1971b; Massey & Ghisla, 1974). Surprisingly, this band was not observed during a recent study of a pig liver enzyme of general specificity (Hall & Kamin, 1975). However, further studies have demonstrated the presence of this band on the addition of octanoyl-CoA (Hall & Lambeth, 1978; Hall, Lambeth, & Kamin, personal communication). In Figure 5, an isosbestic point at 340 nm is retained throughout the titration. The isosbestic point at 499 nm, preserved during the addition of the first equivalent of substrate, is lost at higher concentrations as the long-wavelength band begins to disappear with continuing decline in 446-nm absorption. A sharp break at 1 equiv is not observed in the titration (see inset to Figure 5); however, extrapolation of the linear portions of the curves intersects at approximately 1 mol of octanoyl-CoA/mol of FAD. The percent bleaching at 446 nm on the addition of 13 μM (1 equiv of substrate/FAD) and 100 μM octanoyl-CoA is shown in Table II.

In marked contrast, considerable molar excesses of palmitoyl-CoA produce only a partial bleaching of the flavin chromophore (Figure 6) with the appearance of a weak, featureless, long-wavelength band extending to 740 nm. The decrease in absorption at 446 nm, on the addition of approximately 1 equiv of palmitoyl-CoA (curve 4), is accompanied by a marked red shift of the absorbance maximum (to 455 nm), with the appearance of pronounced shoulders at 480 and 430 nm. These changes suggest that the flavin environment becomes significantly more hydrophobic on binding this long-chain substrate (Palmer & Massey, 1968). Similar spectral perturbations are not observed during octanoyl- and butyryl-CoA titrations (Figures 5 and 7, respectively). After approximately 1 equiv of palmitoyl-CoA, no further red shift or increase in long-wavelength absorption occurs (Figure 6), although the absorbance at 446 nm continues to decline. The changes at 446 nm are slightly sigmoidal (see inset). In connection with this complicated behavior, it should be noted that palmitoyl-CoA has a low critical micelle concentration (Zahler et al., 1968), and micelle formation would be anticipated above 1 equiv of added substrate. Nonspecific binding of palmitoyl-CoA could conceivably perturb the flavin chromophore, in addition to the changes induced by occupation of the CoA–substrate binding site. An inhibitory effect of palmitoyl-CoA micelles on the beef heart long-chain acyl-CoA dehydrogenase has been reported (Hall et al., 1976).

Scheme I

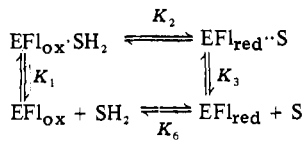
$$\begin{array}{ccccccc} SH_2 + EFl_{ox} & \overset{K_1}{\rightleftharpoons} & EFl_{ox}\cdot SH_2 & \overset{K_2}{\rightleftharpoons} & EFl_{red}\cdot\cdot S & \overset{K_3}{\rightleftharpoons} & EFl_{red} + S \\ \Big\Updownarrow K_4 & & & & & & \Big\Updownarrow K_5 \\ EFl_{ox}\cdot S & & & & & & EFl_{red}\cdot SH_2 \end{array}$$

Little or no long-wavelength absorption is observed on the addition of butyryl-CoA to the kidney enzyme (Figure 7). One equivalent of substrate produces only a 31% decrease in absorbance at 446 nm, but considerably more bleaching is obtained at higher molar excesses (inset). In contrast to octanoyl and palmitoyl substrates, the spectral changes produced by each addition of butyryl-CoA were not over before measurements could be made but were biphasic with the slow phase requiring several minutes for completion. Similar observations were obtained with pig liver general acyl-CoA dehydrogenase (Beinert & Page, 1957). Aerobic butyryl-CoA titrations are complicated, particularly at low substrate levels, by slow reoxidation of the enzyme. For consistency the three titrations described above were performed anaerobically.

The detailed titrations shown in Figures 5–7 are in agreement with previous results for the pig liver general acyl-CoA dehydrogenase (Crane et al., 1956; Beinert & Page, 1957). Addition of reduced substrate to the oxidized dehydrogenase results in the establishment of equilibria (see Scheme I). Both octanoyl- and palmitoyl-CoA produce maximal long-wavelength absorption at approximately 1 equiv of substrate/mol of FAD. Since this band probably represents a charge-transfer interaction between flavin and bound ligand, these results suggest that at 1 equiv of these two substrates the predominant enzyme species are $EFl_{ox}\cdot SH_2$ and $EFl_{red}\cdot\cdot S$ (Scheme I), with little free enzyme forms present. Both octanoyl- and palmitoyl-CoA have been shown to bind very tightly to the pig liver enzyme (Steyn-Parvé & Beinert, 1958). The internal equilibrium (K_2) between these two forms comprising the central complex appears to depend on the hydrocarbon chain length of the bound substrate moiety. With octanoyl-CoA this equilibrium lies to the right, and the observed spectrum is dominated by a reduced flavin–oxidized

substrate component, whereas with palmitoyl-CoA an oxidized flavin–reduced substrate complex is favored.

Since it is reasonable to assume that the redox potentials of the couples *trans*-oct-2-enoyl-/octanoyl-CoA and *trans*-hexadec-2-enoyl-/hexadecanoyl-CoA are similar in free solution, the equilibrium (K_6) should be independent of the chain



length of the acyl-CoA substrate. Thus, differences in K_2 between substrates are primarily a reflection of the affinity of EFl_{ox} for SH_2 and EFl_{red} for S (K_1 and K_3, respectively). At higher substrate concentrations, excess SH_2 begins to compete with S for EFl_{red}, thereby disturbing the equilibrium (K_2) toward further reduction of the flavin (Scheme I). $EFl_{red}\cdot SH_2$ would not be expected to exhibit long-wavelength absorption because the saturated hydrocarbon chain of SH_2 would not be a suitable charge-transfer acceptor moiety. The loss of isosbestic point at 499 nm and the gradual decline in charge-transfer absorption on the addition of excess octanoyl-CoA (Figure 5) are thus consistent with Scheme I.

Butyryl-CoA was shown by Steyn-Parvê & Beinert (1958) to bind much more weakly to the liver general acyl-CoA dehydrogenase than octanoyl-CoA. It is therefore likely that, at the rather low enzyme concentrations used for the titration experiments (approximately 13 μM), appreciable levels of the free species EFl_{ox} and EFl_{red} exist on the addition of 1 equiv of butyryl-CoA. Since the concentration of EFl_{red}·crotonyl-CoA species formed during the titration shown in Figure 7 cannot be evaluated at present, it is uncertain whether this species exhibits long-wavelength absorption of the type shown by EFl_{red}·octenoyl-CoA. It is likely that the structural requirements for effective charge-transfer interaction are stringent, as was demonstrated for the bacterial enzyme by Engel & Massey (1971b).

Acknowledgments

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