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Butyryl-CoA dehydrogenase from *Megasphaera elsdenii*

Specificity of the catalytic reaction

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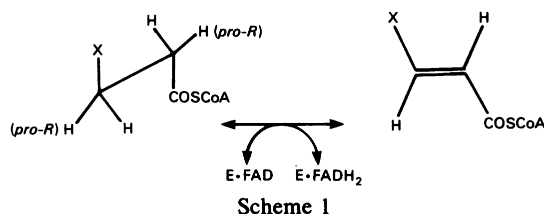
The absorption coefficient of butyryl-CoA dehydrogenase from *Megasphaera elsdenii* at 450nm is determined as $14.4\,mM^{-1}\cdot cm^{-1}$ in the CoA-free form and $14.2\,mM^{-1}\cdot cm^{-1}$ in the CoA-liganded form (both yellow). The latter value is considerably higher than the earlier published estimate. Phenazine ethosulphate offers great advantages over phenazine methosulphate as a coupling dye in the catalytic assay despite giving lower $V_{max.}$ values ($506\,min^{-1}$ as compared with $1250\,min^{-1}$ under the conditions used). The phenazine ethosulphate assay is used to establish a pH optimum of 8.05 for oxidation of 100μM-butyryl-CoA. The rates of oxidation of a range of straight-chain, branched-chain and alicyclic acyl thioesters are used to provide the following information. (1) Only straight-chain acyl groups containing 4–6 carbon atoms are easily accommodated by the postulated hydrophobic pocket of the enzyme. (2) C-3-substituted acyl-CoA thioesters are not oxidized at a significant rate, suggesting that the C-3 *pro-S*-hydrogen atom of straight-chain substrates is partially exposed to the solvent. (3) Acyl-CoA thioesters with substitutions at C-2 are oxidized, though at a lower rate than their straight-chain counterparts. This implies that the C-2 *pro-S*-hydrogen atom of straight-chain substrates is partially exposed to the solvent. (4) Saturated alicyclic carboxylic acyl-CoA thioesters with 4–7 carbon atoms in the ring are oxidized, with maximal activity for the cyclohexane derivative. This implies that optimal oxidation requires a true *trans* orientation of the two departing hydrogen atoms. (5) The strain imposed by bound unsaturated alicyclic acyl thioesters strikingly perturbs the flavin visible-absorption spectrum, with the exception of the cyclohex-2-ene derivative, which forms a complex with similar spectral properties to those of the crotonyl-CoA complex. (6) In the thiol moiety of thioester substrates the amide bond of *N*-acetylcysteamine is essential for both binding and catalysis. The adenosine structure contributes substantially to strong binding, but is less important in determining the catalytic rate.

Butyryl-CoA dehydrogenase (EC 1.3.99.2) catalyses the oxidation of saturated acyl-CoA thioesters to give a 2,3-unsaturated product (Green *et al.*, 1954) by removal of the two *pro-R*-hydrogen atoms (Biellmann & Hirth, 1970*a*,*b*) (Scheme 1).

In vivo, the enzyme interacts with the electron-transferring flavoprotein (Crane & Beinert, 1956; Whitfield & Mayhew, 1974). *In vitro*, electron-transferring protein can be replaced by dye systems such as PMS/DCPIP (Engel & Massey, 1971*a*) or Meldolablau (8-dimethylamino-2,3-benzophenoxazinium chloride)/2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl-2*H*-tetrazolium chloride (Dommes & Kunau, 1976). Both systems have drawbacks: PMS is very unstable, especially in light, and reacts non-enzymically with DCPIP (McIlwain, 1937; King, 1963; Ghosh & Quayle, 1979), whereas a detergent is required to solubilize 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl-2*H*-tetrazolium chloride

Abbreviations used: DCPIP, 2,6-dichlorophenol-indophenol; PES, phenazine ethosulphate; PMS, phenazine methosulphate.



Scheme 1

and the system is less sensitive than that with PMS and DCPIP. An improvement in the assay that allows more reproducible rate measurements is suggested in the present paper.

Ox liver butyryl-CoA dehydrogenase oxidizes acyl-CoA substrates with C_4–C_8 acyl chains and shows optimal activity at C_5 (Shaw & Engel, 1984), whereas the pig liver enzyme oxidizes C_3–C_8 acyl chains with optimal activity at C_4 (Green *et al.*, 1954). Separate dehydrogenases for branched-chain acyl-CoA thioesters have been shown to exist in mammalian systems (Noda *et al.*, 1980). Since butyryl-CoA dehydrogenase from *Megasphaera elsdenii* forms complexes with a wide range of acyl-CoA thioesters (Engel & Massey, 1971*b*), it was of interest to determine its substrate-specificity in the catalytic reaction. We have employed a range of straight-chain, branched-chain and alicyclic acyl-CoA thioesters in order to gain information on the active site and active centre of this bacterial butyryl-CoA dehydrogenase.

Materials and methods

Enzyme

Butyryl-CoA dehydrogenase was prepared by the method of Engel (1981) from *Megasphaera elsdenii* (A.T.C.C. 25940). Tightly bound CoA and CoA persulphide were removed by the method of Williamson & Engel (1982). Enzyme thus treated is referred to as 'CoA-free' and is yellow. Spectra were recorded with a Cary 219 spectrophotometer.

Assay

Butyryl-CoA dehydrogenase was assayed at 25°C in a reaction mixture (1.1 ml) comprising the following: 0.15 M-potassium phosphate buffer, pH 8; 0.001% DCPIP; enzyme and various acyl thioesters (5 μM–5 mM) as required; 1 mM-PES (see below) to start the reaction. The catalytic rate is proportional to the rate of reduction of DCPIP at 600 nm (Engel & Massey, 1971*a*) (ε_{600} = 22 mM^{-1}·cm^{-1}; Armstrong, 1964). Absorbance changes were measured by using either a Cary 219 or a Perkin–Elmer Lambda 1 UV/vis spectrophotometer.

Reagents

Acetic anhydride, guanidinium chloride (AristaR grade), butyric anhydride, DCPIP and ethanethiol were purchased from BDH Chemicals, Atherstone, Warwicks., U.K. Propionic anhydride and 2-mercaptoethylamine hydrochloride were from Koch–Light Laboratories, Slough, Berks., U.K. Glutaric anhydride, CoA, *S*-2-methylbutyric acid, pentanoic acid, hexanoic acid, heptanoic acid, PES and PMS were from Sigma Chemical Co., Poole, Dorset, U.K. 2-Methylpropionic acid was from Fisons Scientific Apparatus, Loughborough, Leics., U.K. Cyclopentanecarboxylic acid and cycloheptanecarboxylic acid were purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Cyclopropanecarboxylic acid and cyclobutanecarboxylic acid were generously given by Dr. H. S. A. Sherratt. 2-Methylpentanoic acid, 3-methylpentanoic acid, 4-methylpentanoic acid, cyclohexanecarboxylic acid and 3-methylbutyric acid were purchased from Eastman–Kodak, Kirkby, Liverpool, U.K. Butyrolactone was from K and K Laboratories, Plainview, NY, U.S.A. Pantethine was from C. P. Laboratories, Bishops Stortford, Herts., U.K.

Preparation of acyl-CoA thioesters

Acetyl-CoA, propionyl-CoA, butyryl-CoA and glutaryl-CoA were made from CoA and the appropriate anhydrides by the method of Simon & Shemin (1953) as modified by Stadtman (1957).

3-Methylbutyryl-CoA, cyclobutanecarboxylic acyl-CoA, cyclopentanecarboxylic acyl-CoA, pentanoyl-CoA, hexanoyl-CoA and heptanoyl-CoA were made by the method of Kawaguchi *et al.* (1981).

2-Methylpropionyl-CoA, *S*-2-methylbutyryl-CoA, *RS*-2-methylpentanoyl-CoA, *RS*-3-methylpentanoyl-CoA, cyclopropanecarboxylic acyl-CoA, cyclohexanecarboxylic acyl-CoA and cycloheptanecarboxylic acyl-CoA were made by the mixed-anhydride method (Goldman & Vagelos, 1961). 3-Hydroxybutyryl-CoA was made from CoA and butyrolactone (Vagelos & Earl, 1959).

Purification of acyl-CoA thioesters

Butyryl-CoA, pentanoyl-CoA, hexanoyl-CoA, heptanoyl-CoA, cyclobutanecarboxylic acyl-CoA and cyclopentanecarboxylic acyl-CoA were purified by using DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) as follows. A 35 cm × 1 cm column containing DEAE-Sephadex A-25 was equilibrated with 0.2 M-NaCl in 0.1 mM-HCl. Freeze-dried acyl-CoA was taken up in 1 ml of water and applied to the column, which was then eluted with a gradient of 0.2–0.6 M-NaCl in 0.1 mM-HCl. Each acyl-CoA gave a main peak centred at 0.36 M-NaCl. Pooled fractions were freeze-dried and NaCl was removed by gel filtration on Bio-Gel P-2, pre-equilibrated in 0.1 mM-HCl.

The other acyl-CoA thioesters were purified by high-pressure liquid chromatography (Applied Chromatography Systems, Luton, Beds., U.K.) on a semi-preparative (250 mm × 8 mm) Spherisorb 5 ODS 2 (C_{18}) column (Hichrom, Reading, Berks., U.K.) with a gradient of 7–70% (v/v) methanol in

10mM-KH_2PO_4 (Corkey *et al.*, 1981). The eluate was monitored at 254nm.

Determination of purity of acyl-CoA thioesters

(i) The A_{232}/A_{260} ratio (Stadtman, 1957) is 0.50–0.54:1 for CoA thioesters and 0.22:1 for free CoA, and thus serves as a convenient indicator of the relative proportions of thioester and nucleotide.

(ii) Analytical high-pressure liquid chromatography was employed to determine the number of components in a given acyl-CoA solution, The system, similar to that described above but with an analytical (250mm × 5mm) Spherisorb 5 ODS 2 (C_{18}) column, indicated that each acyl-CoA was over 95% pure.

Preparation of S-butyrylpantetheine

(+)-Pantethine was converted into pantetheine by reduction of the disulphide bond with $NaBH_4$ (Gomes *et al.*, 1981). The *S*-butyryl derivative was prepared with butyric anhydride (Simon & Shemin, 1953; Stadtman, 1957).

Preparation of NS-dibutyrylcysteamine

This thioester was made by the following modification of the method of Gerstein & Jencks (1964). A solution of 0.5g of 2-mercaptoethylamine hydrochloride in 20ml of water was adjusted to pH9 with 0.1M-NaOH and stirred for 1h. The pH was adjusted to 7.5 with 0.1M-HCl, and a 3-fold molar excess of butyric anhydride was added, slowly and with vigorous stirring. The pH was kept at 7.5 by additions of 1M-NaOH. After a further 15min of stirring, the white precipitate was extracted into diethyl ether. The ether layer was washed with several portions of first 1M-NaCl and then water, and dried over anhydrous Na_2SO_4. The ether was blown off with N_2, and the *NS*-dibutyrylcysteamine was taken up in a small volume of ethanol and freeze-dried.

Preparation of S-butyryl-N-propionylcysteamine

NS-Dipropionylcysteamine was made as described above, but with propionic anhydride instead of butyric anhydride. The *NS*-dipropionylcysteamine was then dissolved in ethanol/water (1:1, v/v). The nitroprusside test (Stadtman, 1957) showed that no free thiol groups were present. NaOH was then added to a final concentration of 2M and the mixture was stirred at 20°C. After 40min, 1M-HCl was added to give pH7.5. The nitroprusside test showed that the thioester bond had been hydrolysed. The resulting *N*-propionylcysteamine was stirred, and butyric anhydride (in a small volume of ethanol) was added until the nitroprusside test became negative. After 15min, the pH was adjusted to 4. The product was extracted, washed, dried and freeze-dried as for *NS*-dibutyrylcysteamine.

Preparation of S-butyryl-N-acetylcysteamine

S-Butyryl-*N*-acetylcysteamine was prepared by the same method as described above, but with acetic anhydride instead of propionic anhydride.

Preparation of S-butyrylethanethiol

A 0.5ml portion of ethanethiol was dissolved in 15ml of ethanol/water (1:1, v/v) and the pH adjusted to approx. 7.5. Then 1ml of butyric anhydride was added, and the mixture was shaken vigorously. Nitroprusside indicated that all of the free thiol groups had reacted. *S*-Butyrylethanethiol was then extracted into diethyl ether and washed with 2vol. of 5% $NaHCO_3$, 2vol. of saturated NaCl and 1vol. of water. After the extract had been dried over anhydrous Na_2SO_4, the ether was blown off to leave the oily thioester.

Determination of the S-butyryl thioesters

(i) Saturated acyl thioesters exhibit a characteristic absorption spectrum with a peak at 230–233nm ($\varepsilon_{233} = 4.0\,\text{mM}^{-1}\cdot\text{cm}^{-1}$; Kass & Brock, 1969).

(ii) Samples of thioester were hydrolysed in 0.2M-NaOH at 20°C for 10min. After neutralization, the amount of released thiol was determined by using 5,5′-dithiobis-(2-nitrobenzoic acid ($\varepsilon_{412} = 13.6\,\text{mM}^{-1}\cdot\text{cm}^{-1}$; Ellman, 1959).

Absorption coefficients

A 1ml sample of an enzyme solution in 0.1M-potassium phosphate buffer, pH7, was placed in one sector of a double-sector glass cuvette (two 0.4cm pathlengths), adjusted to 1ml of 10M-guanidinium chloride in the same buffer. When the spectrum had been recorded, the contents of both sectors were mixed, and the spectrum was recorded again after 10min. The absorption coefficient at 450nm was calculated according to the formula:

$$\varepsilon = \frac{A_{\text{native}}}{A_{\text{denatured}}} \times 11.8\,\text{mM}^{-1}\cdot\text{cm}^{-1}$$

where $11.8\,\text{mM}^{-1}\cdot\text{cm}^{-1}$ is the absorption coefficient of FAD in guanidinium chloride (Thorpe *et al.*, 1979).

Results

Absorption coefficient for 'CoA-free' butyryl-CoA dehydrogenase

Three samples of 'CoA-free' butyryl-CoA dehydrogenase (A_{450} approx. 0.2) were denatured as described in the Materials and methods section. An absorption coefficient of $14.4\,\text{mM}^{-1}\cdot\text{cm}^{-1}$ at 450nm was calculated by this means. Similarly, the value for 'yellow' butyryl-CoA dehydrogenase (i.e.

native enzyme after reduction with dithionite and air re-oxidation) was $14.2\,mM^{-1}\cdot cm^{-1}$. This value is higher than that reported by Engel & Massey (1971*a*), who used trichloroacetic acid precipitation. The discrepancy arises from the method used in that earlier investigation, which failed to take account of the concentrating effect of the ether treatment during extraction of the added trichloroacetic acid. The value reported in the present paper is closer to those obtained by Thorpe *et al.* (1979) for pig kidney general acyl-CoA dehydrogenase ($15.4\,mM^{-1}\cdot cm^{-1}$ at 446nm) and by Shaw & Engel (1984) for ox liver butyryl-CoA dehydrogenase ($14.9\,mM^{-1}\cdot cm^{-1}$ at 448nm).

Intermediate electron carrier

Lineweaver–Burk plots were obtained by varying the concentration of either PMS or PES, in the presence of constant concentrations of butyryl-CoA and DCPIP. The measured enzymic rates showed no lag in either case, and the following kinetic constants were obtained: PES, apparent $K_m = 0.59\,mM$, $V_{max.} = 506\,min^{-1}$; PMS, apparent $K_m = 0.83\,mM$, $V_{max.} = 1250\,min^{-1}$. The points obtained with the PMS-mediated assay showed a much greater scatter, whereas those with PES as the coupling dye gave excellent linearity regardless of the linear graphical transformation employed.

The rates of the non-enzymic reduction of DCPIP by PES and PMS were examined. In a system consisting of 0.001% DCPIP, 0.15M-potassium phosphate buffer, pH7, 0.1mM-butyryl-CoA and various concentrations of PES or PMS, the following changes in A_{600} were observed: PMS, 0.2mM, $0.0015\,min^{-1}$; PMS, 1.0mM, $0.0085\,min^{-1}$; PES, 0.2mM, $0.0002\,min^{-1}$: PES, 1.0mM, $0.0008\,min^{-1}$. The undesirable non-enzymic rate of reduction of DCPIP by PMS is much higher and less reproducible than that shown by PES.

The concentration of PMS (0.13mM) used by Engel & Massey (1971*a*) gave a turnover of approx. $200\,min^{-1}$. However, this is only 16% of the maximum turnover number possible ($1250\,min^{-1}$) when PMS is at a concentration sufficient to saturate the enzyme. The apparent K_m for PMS is 0.8mM, and so 0.13mM-PMS lies on the steep portion of the Michaelis plot. A small change in the final assay concentration of PMS would therefore produce a relatively large change in the rate, a problem that is exacerbated by the inherent instability of PMS. Raising the concentration of PMS to saturating values, however, does not resolve the problem, since an unacceptable rate of non-enzymic reduction of DCPIP results. These observations support the suggestion by Ghosh & Quayle (1979), on the basis of their work with methanol dehydrogenase, that PMS should be replaced by PES whenever possible in dye-linked assays.

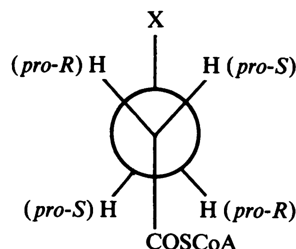
pH optimum of the reaction

The rate of reduction of DCPIP was measured at various pH values, under the following conditions: DCPIP, 0.001%; 0.15M-potassium phosphate buffer, pH as indicated; 10nM-butyryl-CoA dehydrogenase; 0.1mM-butyryl-CoA; 1mM-PES. The rates obtained are expressed as a percentage of the maximum at pH8.05: pH6, 0%; pH7, 25%; pH7.5, 57%; pH7.95, 83%; pH8.05, 100%; pH8.2, 93%; pH8.5, 89%; pH9, 72%. The rate thus falls off more steeply on the acid side of the pH optimum.

The pH optimum of butyryl-CoA dehydrogenase from ox liver (determined with 50μM-butyryl-CoA and also PES and DCPIP) is 7.1 (Shaw & Engel, 1984) and for monkey liver it is 7.4 (Hoskins, 1966). This suggests that the observed optima are properties of the individual enzymes and not merely of the dye-coupling system.

Alkyl-chain specificity

(i) *Straight-chain acyl-CoA thioesters.* The structure of a straight-chain saturated acyl-CoA can be represented by the Newman projection as:



where X = H, CH_3 etc. Increasing the chain length of the acyl group, therefore, increases the size of the alkyl group at position X (*trans* to the CoA moiety in the most stable *anti* conformation).

Table 1 shows the activity of butyryl-CoA dehydrogenase with straight-chain saturated acyl-CoA thioesters. Optimal activity is with the C_4 substrate, butyryl-CoA (X = CH_3). Heptanoyl-CoA is not a substrate, and propionyl-CoA is only a very poor substrate.

(ii) *Branched-chain acyl-CoA thioesters.* Table 1 also shows the activity of butyryl-CoA dehydrogenase towards a number of acyl-CoA thioesters containing substitutions in positions 2, 3 or 4. Only the *S*-isomers allow the correct orientation of the two hydrogen atoms (*pro-R* on carbon atoms with more than one hydrogen atom) that are removed during catalysis (Biellmann & Hirth, 1970*a*,*b*). The preparations of 2-methylpentanoyl-CoA and of 3-methylpentanoyl-CoA used in the present work are

Table 1. *Reactivity of butyryl-CoA dehydrogenase with straight and branched-chain acyl-CoA thioesters*
The assay was performed as described in the Materials and methods section. Double-reciprocal plots of four to eight duplicate rates were used to obtain the stated values.

	Acyl-CoA substitutions at: C-2 'pro-S'	Acyl-CoA substitutions at: C-3 'pro-S'	X (*trans* to CoA moiety)	Apparent K_m (μM)	$V_{max.}$ (min^{-1})	Relative $V_{max.}$ (butyryl-CoA ≡ 100)
Propionyl-CoA	H	H	H	130	1.6	0.5
Butyryl-CoA	H	H	CH_3	16.4	331	100
Pentanoyl-CoA	H	H	CH_2CH_3	15.0	149	45
Hexanoyl-CoA	H	H	$[CH_2]_2CH_3$	53	14	4.2
Heptanoyl-CoA	H	H	$[CH_2]_3CH_3$	–	0	0
2-Methylpropionyl-CoA	CH_3	H	H	161	5.1	1.5
S-2-Methylbutyryl-CoA	CH_3	H	CH_3	213	20	6.0
3-Methylbutyryl-CoA	H	CH_3	CH_3	–	0	0
2-Methylpentanoyl-CoA	CH_3	H	CH_2CH_3	–	0.3*	0.1*
3-Methylpentanoyl-CoA	H	CH_3	CH_2CH_3	–	0	0
4-Methylpentanoyl-CoA	H	H	$CH(CH_3)_2$	97	60	18
Glutaryl-CoA	H	H	CH_2CO_2H	–	0	0
3-Hydroxybutyryl-CoA	H	OH	CH_3	–	0	0

* This was a single rate measurement, at a substrate concentration of 0.44mM.

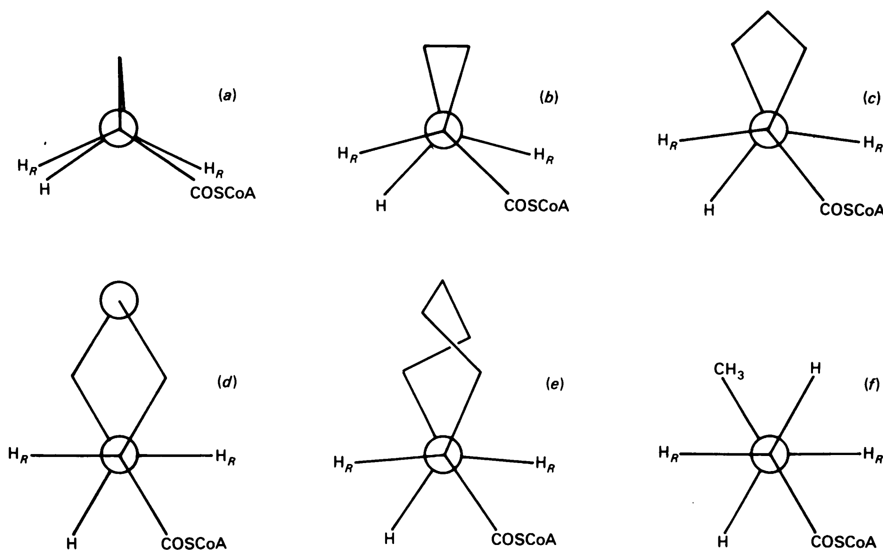


Fig. 1. *Newman projections along the C-2–C-3 axis of various acyl-CoA thioesters*
Each structure is drawn from models by looking along the C-2–C-3 axis. The COSCoA moiety is in the least-hindered quasi-equatorial position. θ is the approximate angle, as viewed along the C-2–C-3 axis, between the two hydrogen atoms that are removed (designated as H_R). (*a*) Cyclopropanecarboxylic acyl-CoA, θ approx. 130°. (*b*) Cyclobutanecarboxylic acyl-CoA, θ approx. 150°; the cyclobutane ring is bent (or folded) with a dihedral angle of 25–30°. (*c*) Cyclopentanecarboxylic acyl-CoA, θ approx. 165°; the cyclopentane ring is puckered, with the C-2 atom out of the plane of the other carbon atoms. (*d*) Cyclohexanecarboxylic acyl-CoA, θ approx. 180°; the chair conformation is the most stable of the alicyclic rings. (*e*) Cycloheptanecarboxylic acyl-CoA, θ approx. 170°; the most stable conformation of the cycloheptane ring contains an axis, but not a plane, of symmetry. (*f*) Butyryl-CoA is shown in its most stable, *anti*, conformation with θ approx. 180°.

racemic mixtures of the *R*- and *S*-forms. In Table 1, therefore, the substitutions shown are derived from the *S*-isomers of these compounds.

4-Methylpentanoyl-CoA, *S*-2-methylbutyryl-CoA and 2-methylpropionyl-CoA are all reasonable substrates for butyryl-CoA dehydrogenase. It

should be noted that glutaryl-CoA and 3-methylbutyryl-CoA are not oxidized at measurable rates under these conditions of assay.

(iii) *Alicyclic acyl-CoA thioesters.* Fig. 1 shows the structures of the alicyclic acyl-CoA thioesters used in the present work. Each is drawn in the most stable conformation for a substituted alicyclic ring (see Roberts & Caserio, 1967; Hendrickson *et al.*, 1970).

Table 2 shows the rates of reaction of the alicyclic acyl-CoA substrates in the assay with PES as electron acceptor. Cyclopropanecarboxylic acyl-CoA is not a substrate. The other alicyclic acyl-CoA thioesters are oxidized, with maximal activity

Table 2. *Reactivity of butyryl-CoA dehydrogenase with alicyclic acyl-CoA thioesters*
The stated values were obtained as described in the legend to Table 1.

Acyl-CoA	Apparent K_m (μM)	$V_{max.}$ (min^{-1})	Relative $V_{max.}$ (butyryl-CoA ≡ 100)
Cyclopropanecarboxylic acyl-CoA	–	0	0
Cyclobutanecarboxylic acyl-CoA	119	3.2	1.0
Cyclopentanecarboxylic acyl-CoA	95	7.0	2.1
Cyclohexanecarboxylic acyl-CoA	82	22	6.7
Cycloheptanecarboxylic acyl-CoA	198	10.6	3.2

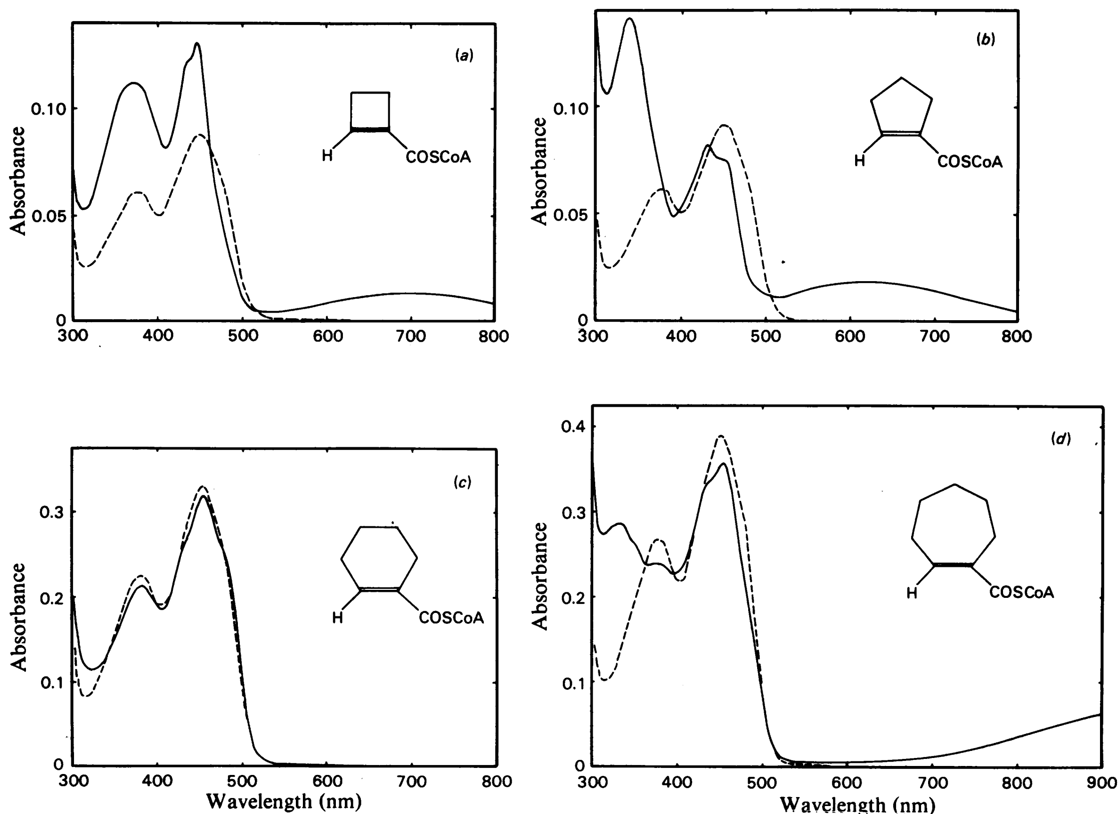


Fig. 2. *Absorption spectra of oxidized butyryl-CoA dehydrogenase–2,3-unsaturated alicyclic acyl-CoA complexes*
The broken lines (----) indicate the initial absorption spectrum of CoA-free butyryl-CoA dehydrogenase. The following alicyclic acyl-CoA compounds were then added aerobically (molar excess shown in parentheses) and spectra (——) recorded after the indicated time: (*a*) cyclobutanecarboxylic acyl-CoA (6.5-fold), 5 min; (*b*) cyclopentanecarboxylic acyl-CoA (6.5-fold), 5 min; (*c*) cyclohexanecarboxylic acyl-CoA (1.2-fold), 10 min; (*d*) cycloheptanecarboxylic acyl-CoA (2-fold), 120 min. In each case no further change on incubation was seen. The insets show the structures of the appropriate 2,3-unsaturated alicyclic acyl-CoA compounds.

Table 3. *Reactivity of butyryl-CoA dehydrogenase with butyryl thioesters of CoA model compounds*
The stated values were obtained as described in the legend to Table 1.

Acyl-CoA	Apparent K_m (μM)	$V_{max.}$ (min^{-1})	Relative $V_{max.}$ (butyryl-CoA ≡ 100)
S-Butyrylethanethiol	–	0	0
S-Butyryl-*N*-acetylcysteamine	3900	6.3	2
S-Butyryl-*N*-propionylcysteamine	948	27	8
NS-Dibutyrylcysteamine	578	22	7
S-Butyrylpantetheine	243	169	51
Butyryl-CoA	16.4	331	100

for the cyclohexane derivative. Examination of Fig. 1 reveals that only in the cyclohexane derivative is the angle θ between the two H_R atoms exactly 180° along the direction of the C-2–C-3 bond, as in butyryl-CoA.

Complexes between butyryl-CoA dehydrogenase and unsaturated alicyclic acyl-CoA thioesters

Fig. 2 shows the visible-absorption spectrum of butyryl-CoA dehydrogenase after aerobic addition of saturated alicyclic acyl-CoA thioesters. Under these conditions molecular O_2 can act as an electron acceptor (Engel & Massey, 1971*a*,*b*) to produce a complex between oxidized enzyme and oxidized (2,3-unsaturated) product. Thus cyclobutanecarboxylic acyl-CoA, on addition to butyryl-CoA dehydrogenase under aerobic conditions, is converted within a few seconds into a cyclobut-2-enecarboxylic acyl-CoA–oxidized butyryl-CoA dehydrogenase complex (Fig. 2*a*). The absorption coefficients of the two flavin peaks are dramatically increased from 11.4 to 21.6mM^{-1}·cm^{-1} (at 378nm) and from 14.4 to 25.2mM^{-1}·cm^{-1} (at 450nm). A long-wavelength band centred at 710nm is also seen (ε_{710} = 3.4mM^{-1}·cm^{-1}).

The complex between cyclopent-2-enecarboxylic acyl-CoA and oxidized butyryl-CoA dehydrogenase also exhibits marked changes when compared with CoA-free butyryl-CoA dehydrogenase (Fig. 2*b*). The appearance of a long-wavelength absorption band centred at 630nm (ε_{630} = 3.0mM^{-1}·cm^{-1}) is accompanied by a large increase in A_{340} (ε_{340} = 20.0mM^{-1}·cm^{-1}), a 20nm blue-shift in the main flavin peak and a pronounced shoulder at approx. 448nm. In marked contrast, the cyclohex-2-enecarboxylic acyl-CoA–oxidized butyryl-CoA dehydrogenase complex shows some resolution in the main flavin peak with a small shoulder at approx. 475nm, but no other marked spectral changes. It should be noted that the visible-absorption spectrum is almost identical with that of the corresponding complex with crotonyl-CoA (Engel & Massey, 1971*b*).

The cyclohept-2-enecarboxylic acyl-CoA–oxidized butyryl-CoA dehydrogenase complex exhibits a long-wavelength band at >900nm (ε_{900} = 2.6mM^{-1}·cm^{-1}) together with some resolution of the main flavin peak and the appearance of an additional peak at 330nm (ε_{330} = 10.5mM^{-1}·cm^{-1}).

Specificity for S-butyryl thioesters of CoA model compounds

Table 3 shows the rates of oxidation, catalysed by butyryl-CoA dehydrogenase, of the *S*-butyryl thioesters. Ethanethiol, *N*-acetylcysteamine, *N*-propionylcysteamine and pantetheine are all fragments of the CoA molecule. Both the rate of oxidation and the apparent affinity increase with increasing size from the ethanethiol derivative (not a substrate) to the CoA derivative (the best substrate). *N*-Butyrylcysteamine is not a true CoA fragment, but has the –NH– group of the β-alanine portion replaced by a terminal methyl group. Although the rate of oxidation of the *S*-butyryl thioester of this compound is slightly lower than that of the *N*-propionyl derivative, the apparent affinity is higher.

Discussion

Acyl moiety

In the homologous series of straight-chain 2,3-unsaturated acyl-CoA thioesters, only pent-2-enoyl-CoA gives rise to a long-wavelength absorption band (Engel & Massey, 1971*b*) in its complex with the oxidized enzyme. Engel (1972) suggested the existence of a hydrophobic pocket of limited size, able to accommodate a part of the acyl chain. The size of this pocket would therefore define the chain-length-specificity of the enzyme. Presumably the acyl chain of heptanoyl-CoA is too large to be accommodated, whereas 4-methylpentanoyl-CoA is a better substrate than hexanoyl-CoA, even though both contain six carbon atoms in the acyl chain. Possibly the acyl chain of propionyl-CoA is too small to interact with the postulated pocket, and hence the correct orientation within the active site cannot be achieved.

The branched-chain substrates provide further insight into the orientation of bound acyl-CoA thioesters. Compounds with a substitution at C-3 (3-methylbutyryl-CoA, 3-methylpentanoyl-CoA

and 3-hydroxybutyryl-CoA) are not significantly oxidized in the assay. This suggests that in the case of butyryl-CoA and other straight-chain substrates the C-3 *pro-S*-hydrogen atom is buried. From the most favourable conformation of butyryl-CoA (see Fig. 1), this arrangement would suggest that the C-2 *pro-S*-hydrogen atom is partially accessible to the solvent. This is supported by the following two observations. (i) Compounds with a substitution at C-2 are substrates (2-methylpropionyl-CoA, *S*-2-methylbutyryl-CoA and 2-methylpentanoyl-CoA), although the activities with the butyryl and pentanoyl derivatives are less than with the corresponding straight-chain compounds. (ii) Some alicyclic acyl-CoA thioesters are also reasonable substrates, and Fig. 1 suggests that the bound alicyclic ring is positioned so that it faces away from the active site, with the C-3 *pro-S*-hydrogen atom buried.

The trend in rates of oxidation of the alicyclic compounds, which peaks with the cyclohexane derivative, suggests that steric hindrance might not be the only factor dictating the rate of the reaction. Fig. 1 shows that the six-membered ring is the only one that favours a 180° *trans* arrangement of the two H_R atoms, as in the straight-chain substrates. In order to achieve this in the case of the other ring systems, some strain has to be introduced. This would tend to increase the required activation energy and decrease the overall rate. Furthermore, a four-membered ring already possesses bond-angle strain of the order of 110kJ/mol greater than the six-membered ring.

The appearance of the absorption spectra of the complexes between oxidized enzyme and the 2,3-unsaturated alicyclic acyl-CoA compounds, which confirm and extend the findings briefly reported by Engel (1972), also reveals a similar trend. The cyclohexane derivative produces only a shoulder at 475nm, indicative merely of a change in the FAD environment to a more hydrophobic one (Harbury *et al.*, 1959; Massey & Ganther, 1965). This is similar to the spectral changes on binding crotonyl-CoA (Engel & Massey, 1971*b*). In contrast, the complexes with other unsaturated alicyclic derivatives all show very much more pronounced spectral changes, with long-wavelength absorption bands beyond 500nm, probably due to charge-transfer (Williamson *et al.*, 1982). The four-membered ring containing the double bond in cyclobut-2-ene-carboxylic acyl-CoA gives rise to substantial bond-angle strain. This ligand produces the most striking change in the two flavin peaks, implying a substantial alteration in the environment of the bound FAD.

CoA moiety

The specificity of butyryl-CoA dehydrogenase for the thioesters of CoA fragments appears to be fairly broad. *S*-Butyryl-*N*-acetylcysteamine is a substrate, although it lacks both the pantoic acid and the adenosine moieties. The large difference in apparent K_m and small difference in $V_{max.}$ for *S*-butyrylpantetheine and for butyryl-CoA suggest that the adenosine moiety is important for tightness of binding, but less important in determining the overall catalytic rate. The presence of the amide bond in *S*-butyryl-*N*-acetylcysteamine appears to be essential for both binding and catalysis, since *S*-butyrylethanethiol is not a substrate.

Relationship between function and specificity

The acyl-chain-length specificity of the bacterial butyryl-CoA dehydrogenase is narrower than that of the corresponding mammalian enzymes (see Green *et al.*, 1954; Beinert, 1963; Shaw & Engel, 1984). Furthermore, 3-hydroxybutyryl-CoA, 3-methylbutyryl-CoA and glutaryl-CoA are not oxidized at appreciable rates in the catalytic assay by either the mammalian or bacterial enzymes, and separate enzymes have been isolated in mammals for these functions (Wakil *et al.*, 1954; Noda *et al.*, 1980). For the bacterial enzyme, however, it should be noted that a feeble activity ($<0.01\,min^{-1}$) towards 3-hydroxybutyryl-CoA and 3-methylbutyryl-CoA was detected by Engel & Massey (1971*a*,*b*) with prolonged incubations of enzyme with stoicheiometric amounts of the acyl-CoA. The low reactivity of the bacterial butyryl-CoA dehydrogenase with propionyl-CoA is perhaps surprising. Although most of the corresponding mammalian enzymes do not catalyse this reaction, it was thought that, *in vivo*, the bacterial enzyme catalysed the expulsion of reducing equivalents by production of propionate as well as butyrate, pentanoate and hexanoate (Baldwin & Milligan, 1964). The possibility that a separate propionyl-CoA dehydrogenase or acrylyl-CoA reductase is present in *M. elsdenii* needs to be plored.

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