The enzymology of dicarboxylic acid formation by *Corynebacterium* sp. strain 7E1C grown on n-alkanes

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Cultures of the Gram-positive bacterium *Corynebacterium* sp. strain 7E1C contained up to 300 mg dodecanedioic acid l⁻¹ after growth on dodecane. Small amounts of tetradecanedioic acid (17 to 45 mg l⁻¹) were produced during growth on tetradecane or methyl tetradecanoate. No dicarboxylic acids were detected after growth on hexadecane, hexadecanoic acid or 16-hydroxyhexadecanoic acid. Studies on the rates of degradation of exogenous dicarboxylic acids showed that this is not a significant factor influencing the accumulation of dodecanedioic and tetradecanedioic acids. The activities and substrate specificities of a number of enzyme activities involved in dicarboxylic acid metabolism were investigated. The specificities of the long-chain acyl-CoA synthetase and thioesterase, alcohol dehydrogenases and β -oxidation are consistent with the accumulation of dodecanedioic acid from dodecane and the lack of production of hexadecanedioic acid from hexadecane. The ω -hydroxy fatty acid may occupy a pivotal position in determining whether significant production of dicarboxylic acid occurs with this organism.

Introduction

Certain yeasts and bacteria produce long-chain dicarboxylic acids from alkanes, n-alkanols and fatty acids both during growth on these substrates and from resting cells presented with these substrates (Bacchin *et al.*, 1974; Kester & Foster, 1963; Shiio & Uchio, 1971; Uchio & Shiio, 1972*a, b, c*; Yi & Rehm 1982*a, b, c*, 1988*a, b*). There has been much industrial interest in microbial dicarboxylic acid production (e.g. Casey & Macrae, 1992; Casey *et al.*, 1990) and numerous dicarboxylic-acid-producing microbes have been patented (see Buhler & Schindler, 1984).

To date, most of the commercial work on microbial dicarboxylic acid metabolism has concentrated on random mutagenesis to produce mutants giving increased dicarboxylic acid yields (Hill *et al.*, 1986; Casey *et al.*, 1990; Furukawa *et al.*, 1986). These mutants are presumably blocked in β -oxidation. Other physiological studies have aimed at optimizing either culture condi-

tions (e.g. Uchio & Shiio, 1972*a*) or the condition of the cells to which the alkane is presented (Chan *et al.*, 1991). Working entirely with whole cells, Yi & Rehm (1982*a, b, c*) demonstrated that dicarboxylic acids can be formed from alkanes via both the α , ω -diol and fatty acid routes (see Fig. 3). In *Candida tropicalis*, the fatty acid route is the major pathway (Yi & Rehm, 1982*c*).

Despite the enzymology of monoterminal alkane oxidation (i.e. alkane to fatty acid) having been investigated in considerable detail (for reviews see Boulton & Ratledge, 1984; Buhler & Schindler, 1984; Rehm & Reiff, 1981), there has been little published on the enzymology of dicarboxylic acid metabolism. Modrzakowski & Finnerty (1989) reported the induction of a dicarboxyl-CoA synthetase in *Acinetobacter* sp. HO1-N grown on one of four alkyl ethers, which were degraded via the corresponding dicarboxylic acid. Activation of a range of dicarboxylic acids from malonate to sebacate was examined and, as these activities varied according to the chain-length of the diethyl growth substrate, it could be that more than one acyl-CoA synthetase was being expressed in the organism. Growth of the bacterium on hexadecane was less effective in inducing activity of the diacyl-CoA synthetase. Yi & Rehm (1988*a*) examined the degradation of 1,18-octadec-9-enoic acid (formed from oleic acid) by whole cells of *C. tropicalis* and observed the formation of a series of unsaturated dicarboxylic acids down to hexenedioic acid that were presumed to arise by direct β -oxidation.

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Abbreviations: DC_n, dicarboxylic acid with a chain-length of *n* carbon atoms; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DMSO, dimethylsulphoxide; PMS, phenazine methosulphate; DCPIP, 2,6-dichlorophenolindophenol.

There have been two studies in which investigations of dicarboxylic acid production by whole cells have been accompanied by enzymological studies. Blasig *et al.* (1988) found that when membrane preparations of the yeast *Candida maltosa* (formerly *Lodderomyces elongisporus*) were incubated with hexadecane the diterminal oxidation product, hexadecanedioic acid, was a minor (10%) oxidation product and monoterminally attack predominated. This correlated well with whole cell studies (Blasig *et al.* 1984). Zhihua & Xiuzhen (1986) reported, somewhat surprisingly, a higher activity of dodecanedioyl-CoA synthase in acetate-grown cells of *Candida tropicalis* than in cells grown on long-chain alkanes. A single, broad-specificity acyl-CoA synthetase may be indicated here, as acetyl-CoA synthetase was similarly induced in cells grown on alkanes or acetate. Mutants of this yeast have been produced showing increased accumulation of hexadecanedioic acid (Yuantong & Xiuzhen, 1988).

In the present work, we have investigated the enzymology of dicarboxylic acid metabolism, the aim being to identify the enzymes that determine the range of dicarboxylic acids that are produced. Such information is vital if a rational approach is to be taken to the problem of increasing dicarboxylic acid yields and manipulating the microbe to produce the dicarboxylic acids of greatest commercial value by, for example, the use of recombinant DNA technologies. The bacterium *Corynebacterium* sp. strain 7E1C was chosen since, of a range of bacteria tested, it produced the most dicarboxylic acids (Broadway, 1990). Furthermore, this is one of the few bacteria in which dicarboxylic acid production has been investigated in any detail (Kester & Foster, 1963; Bacchin *et al.*, 1974; Du Pont 1973, 1974). A bacterium, rather than a yeast, was selected since the lack of subcellular compartmentalization is likely to provide the simpler experimental system.

Methods

Growth of the bacterium. *Corynebacterium* sp. strain 7E1C (ATCC 19067) was grown for 24 h on nutrient broth and then transferred to Jay's/succinate medium in a vortex-stirred bottle as described earlier (Broadway *et al.*, 1992). Cells were harvested at the mid-exponential phase of growth. Cell-free extracts were prepared as described previously (Broadway *et al.*, 1992). A mutant of this organism, *Corynebacterium* ATCC 21747, which produces increased amounts of dicarboxylic acids (Du Pont, 1973, 1974) was also examined.

Enzyme assays

β -Oxidation. β -Oxidation was measured in 'linked' and 'direct' assays as described previously (Broadway *et al.*, 1992).

Alcohol dehydrogenase. Activity was assayed by following NAD(P)⁺ reduction fluorimetrically, essentially as described by Dalziel (1962). The standard assay contained, in 4 ml: 3.1 mmol KH₂PO₄/K₂HPO₄

buffer, pH 8.0, or 0.2 mmol glycine/NaOH buffer, pH 10.0, containing 3.1 mmol Na₂SO₄, 1 μ mol NAD(P)⁺ and cell-free extract. The reaction was initiated by adding the substrate, either dodecanediol (0.27 μ mol), octan-1-ol (1.3 μ mol) or 12-hydroxylaurate (1.3 μ mol), in 20 μ l DMSO. When assaying for the presence of dye-linked alcohol dehydrogenase activities, NAD(P)⁺ was replaced by PMS (0.1 mM) and DCPIP (0.05 mM). The decrease in A_{600} was followed spectrophotometrically ($\epsilon_{600} = 21.85 \text{ mm}^{-1} \text{ cm}^{-1}$ at pH 8.0).

Acyl-CoA synthetase. Activity was assayed by following the disappearance of free CoA-SH on incorporation into acyl-CoA esters (Webster, 1969; Vamecq *et al.*, 1985). The assay contained, in a final volume of 190 μ l: 120 nmol CoA-SH (trilithium salt); 750 nmol ATP (disodium salt); 750 nmol MgCl₂·6H₂O; 24 μ mol KH₂PO₄/K₂HPO₄ buffer, pH 8.0; and 450 nmol fatty acid substrate, introduced into the assay in 6 μ l DMSO. The reaction was initiated by adding the cell-free extract and terminated by adding trichloroacetic acid (60 μ l; 15%, w/v). Protein was removed by centrifugation. Supernatant (200 μ l) was added to 0.8 ml 0.8 M-Tris/HCl buffer, pH 8, containing 250 nmol DTNB and the A_{412} was measured against an aliquot from a blank reaction from which the fatty acid substrate was omitted ($\epsilon_{412} = 13.6 \text{ mm}^{-1} \text{ cm}^{-1}$ for the thiol anion of DTNB).

Thioesterase. This was assayed continuously by following the increase in A_{412} on reaction of released CoA-SH with DTNB. The assays contained, in 0.9 ml: 130 μ mol KH₂PO₄/K₂HPO₄ buffer, pH 8.0; 0.25 μ mol DTNB; and cell-free extract. The reaction was initiated by adding of 72 nmol acyl-CoA.

'NADH oxidase'. Activity was assayed spectrophotometrically by following the decrease in A_{340} on oxidation of NADH, $\epsilon_{340} = 6.22 \text{ mm}^{-1} \text{ cm}^{-1}$. Assays contained, in 0.9 ml: 130 μ mol KH₂PO₄/K₂HPO₄ buffer, pH 8.0, and 16.5 or 33 nmol NADH. The reaction was initiated by adding the cell-free extract.

Dicarboxylic acid production from different carbon sources. Bacteria were grown on nutrient broth as described above: 1 ml was transferred to Jay's medium (100 ml) in a 250 ml conical flask supplemented with the appropriate carbon source (2% w/v). The cultures were then incubated for 120 h on a rotary shaker (160 r.p.m.) at 30 °C.

Extraction of dicarboxylic acids. Entire cultures (100 ml) were acidified to pH 1 to 2 with conc. H₂SO₄. An internal standard (DC₁₃, 10 mg) was added and the culture was extracted twice with an equal volume of diethyl ether. The ether layers were combined, washed with distilled water (0.1 vol.), and the ether layer was recovered and evaporated to dryness in a rotary evaporator at 30 °C. The residue was dried *in vacuo* over P₂O₅ at 55 °C. The dried residue was dissolved in methanol (5 ml), methylated with trimethylsulphonium hydroxide (Butte, 1983) and a 1 μ l vol. was analysed by GLC.

Degradation of dicarboxylic acids by resting cells. Cells were grown and harvested as described above, except that sterile buffer and centrifuge tubes were used throughout. Whole-cell suspension [5 ml, approx. 9 mg dry wt ml⁻¹] was aseptically transferred to 40 ml 100 mM-KH₂PO₄/K₂HPO₄ buffer, pH 7.0, in a 100 ml conical flask containing dicarboxylic acid (80 mg). The flasks were incubated at 30 °C on a rotary shaker (160 r.p.m.) for 18 to 120 h. Incubations were terminated by acidification to pH 1 to 2 with conc. H₂SO₄; a dicarboxylic acid internal standard (10 mg) was then added and the culture medium was extracted with diethyl ether. For DC₁₆ incubations, the internal standard was DC₁₃. For all other incubations, the internal standard was the dicarboxylic acid two carbons longer than the test dicarboxylic acid.

GLC conditions. A Pye Unicam PU4500 Chromatograph fitted with a flame ionization detector in conjunction with a Pye Unicam CDP4 computing integrator was used with a glass column (1.75 m × 4 mm internal diameter) packed with 10% diethyleneglycol succinate on

Celite, 100–120 mesh. The column was maintained at 200 °C with injection and detection ports at 220 °C. The carrier gas was N₂ supplied at 35 ml min⁻¹.

Synthesis of acyl-CoA esters. The acyl-CoA esters of ω -hydroxyfatty acids and of dicarboxylic acids were synthesized and purified as described previously (Broadway *et al.*, 1992).

Results

Range of carboxylic acids produced

Dicarboxylic acid production from C₁₀ to C₁₆ alkanes and fatty acid methyl esters (FAMES) used as growth substrates for *Corynebacterium* 7E1C were investigated (Table 1). Dodecane gave the highest yield of a dicarboxylic acid (DC₁₂). Small amounts of DC₁₂ were also produced from methyl laurate (despite the lack of growth on this as carbon source), probably due to transformation of the growth substrate by cells present in the initial inoculum. Some DC₁₄ was produced from C₁₄ substrates. No dicarboxylic acids were detected from either hexadecane, methyl palmitate or 16-hydroxypalmitate. Essentially the same range of dicarboxylic acids was produced by *Corynebacterium* sp. ATCC 21747, a mutant derived from *Corynebacterium* sp. strain 7E1C and which produces dicarboxylic acids in increased amounts (Du Pont 1973, 1974). Dicarboxylic acid production in the mutant was increased 3- to 4-fold as compared to *Corynebacterium* sp. strain 7E1C. The spectrum of dicarboxylic acids produced by *Corynebacterium* sp. strain 7E1C was similar to that obtained by Kester & Foster (1963), who observed dicarboxylic acid production from C₁₁ to C₁₄ alkanes with dodecane and tridecane being the optimal substrates.

We have not studied fatty acid production from growth on n-alkanes in any detail, but some observations are worth noting. Analysis of total fatty acids (free and esterified) from cells grown on hexadecane showed that the major product was palmitate (47% of the total). Tetradecanoate (6%), dodecanoate (18%) and decanoate (12%) were also found. No dicarboxylic acids were produced (Table 1). After growth on dodecanol, the products were laurate (15%), DC₁₂ (23%), DC₈ + DC₁₀ (~2%), palmitate (1.4%) and a major (~40%) unidentified compound. The latter had a longer retention time on GLC than DC₁₂ and might have been a product of β -oxidation of DC₁₂ (perhaps 3-keto DC₁₂). This unidentified material was not seen at all on growth on hexadecane.

Degradation of dicarboxylic acids by resting cells

The breakdown and metabolism of exogenous DC₆ to DC₁₆ acids by washed cells of *Corynebacterium* sp. strain 7E1C grown on succinate was investigated (Table 2).

Degradation proceeded via a homologous series of DC_n with chain shortening by multiples of two carbon units consistent with β -oxidation. β -Oxidation is constitutive in this organism (see below) and the results are therefore relevant to DC_n production by alkane-grown cells. DC₆ and DC₈ were attacked rather slowly and DC₁₆ was

Table 1. *Dicarboxylic acid (DC) production by Corynebacterium* 7E1C during growth on a range of carbon sources

Bacteria were grown on the appropriate carbon source for 5 d and then the entire culture was extracted with diethyl ether. No growth was observed on either decane or methyl decanoate and no dicarboxylic acids were detected.

Growth substrate	DC produced (mg ml ⁻¹):			
	DC ₁₀	DC ₁₂	DC ₁₄	DC ₁₆
Dodecane (C ₁₂)	12	301	ND	ND
Methyl laurate (C ₁₂)*	ND	24	ND	ND
Tetradecane (C ₁₄)	10	6	17	ND
Methyl myristate (C ₁₄)	2	2	45	ND
Hexadecane (C ₁₆)	ND	ND	ND	ND
Methyl palmitate (C ₁₆)	ND	ND	ND	ND
16-Hydroxypalmitate (C ₁₆)	ND	ND	ND	ND

ND, Not detected.

* No growth occurred on methyl laurate; production of DC₁₂ was probably due to transformation of the growth substrate by cells in the inoculum.

Table 2. *Degradation of dicarboxylic acids (DC) by washed whole cells of succinate-grown Corynebacterium* 7E1C

Washed whole cells (5 ml, cell density as indicated) were incubated for the time shown at 30 °C on a shaker (160 r.p.m.).

Parent DC (80 mg)	DC detected in incubation medium (mg)					
	DC ₁₆	DC ₁₄	DC ₁₂	DC ₁₀	DC ₈	DC ₆
(a) 8.1 mg dry wt ml ⁻¹ , 18 h						
DC ₁₆	39.6		1.4	0.5	2.1	0.2
DC ₁₄		12.5	5.1	6.8	4.7	0.2
DC ₁₂			12.3	7.4	5.1	1.2
DC ₁₀				0.7	3.7	14.7
DC ₈					47.8	0
DC ₆						43.5
(b) 9.2 mg dry wt ml ⁻¹ , 30 h						
DC ₁₆	17.3	0	0	2.1	0.9	0
DC ₁₄		0	0	13.6	32.6	2.3
DC ₁₂			14	20.9	18.8	6
DC ₁₀				3	19.2	7.2
DC ₈					42.0	0.6
DC ₆						42.8
(c) 9 mg dry wt ml ⁻¹ , 120 h						
DC ₁₆	1	0	0	0	0	0
DC ₁₄		0	0	0	0	0
DC ₁₂			0	0	0.6	0.8
DC ₁₀				0	0.7	3.3
DC ₈					14.9	44.9

Table 3. β -Oxidation of monocarboxylic, ω -hydroxymonocarboxylic and dicarboxylic acids by a crude cell-free extract of succinate-grown *Corynebacterium* 7E1C

Activity was measured as NAD⁺ reduction in the linked assay system of Broadway *et al.* (1992) in potassium phosphate buffer (130 mM, pH 8.0).

Substrate	β -Oxidation activity [nmol min ⁻¹ (mg protein) ⁻¹]
Palmitate (C ₁₆)	38.9
Myristate (C ₁₄)	30.9
Laurate (C ₁₂)	28.9
Decanoate (C ₁₀)	8.3
DC ₁₆	< 1.0
DC ₁₄	2.9
DC ₁₂	1.9
DC ₁₀	1.1
16-Hydroxypalmitate (C ₁₆)	45.0
12-Hydroxylaurate (C ₁₂)	8.4

degraded to a lesser extent than DC₁₀, DC₁₂ or DC₁₄. However DC₁₆, unlike the other compounds used, formed a precipitate in the incubation mixture thereby decreasing its availability to the cells.

β -Oxidation by cell-free extracts

Two assays were used to follow β -oxidation. One was a linked assay in which the acyl-CoAs were generated *in situ* by synthetases present in the crude extracts. The other was a direct method and used chemically synthesized acyl-CoAs. The linked assay could be used only with extracts from succinate-grown cells, whereas the direct assay could be used with extracts from cells grown on various carbon sources (Broadway *et al.*, 1992). For both assays, activity was measured by either NAD⁺ reduction or acetyl-CoA formation. In direct assays, the rates of acetyl-CoA formation and NAD⁺ reduction were in the ratio of approximately 1:1, with lauroyl-CoA, 12-hydroxylauroyl-CoA and 16-hydroxypalmitoyl-CoA as substrates. This is as expected for β -oxidation and showed that the alcohol dehydrogenase activity that was present in cell extracts did not interfere significantly with assays of β -oxidation of hydroxy fatty acids monitored by NAD⁺ reduction.

β -Oxidation activity in *Corynebacterium* sp. strain 7E1C was constitutive, being expressed at similar specific activities in cells grown on glucose, succinate, 1,12-dodecanediol, hexadecanol or methyl palmitate. Using myristoyl-CoA in direct assays, the specific activities were 41 ± 6 nmol min⁻¹ (mg protein)⁻¹ with the carbon sources indicated (Broadway, 1990). In *Escherichia coli*, by contrast, the β -oxidation system is induced by growth

Fig. 1. Substrate specificity of β -oxidation in extracts of hexadecanol-grown and succinate-grown *Corynebacterium* 7E1C. Activities were measured in direct assays in potassium phosphate buffer, pH 8.0, as described in the text. ○, Succinate-grown cells; 100% = 60 nmol min⁻¹ (mg protein)⁻¹. ●, Hexadecanol-grown cells; 100% = 52 nmol min⁻¹ (mg protein)⁻¹. [100% refers to the activity observed with myristoyl-SCoA (C₁₄).]

Table 4. β -Oxidation of monocarboxyl-, ω -hydroxymonocarboxyl- and dicarboxyl-CoA esters by cell-free extracts of *Corynebacterium* 7E1C grown on a range of carbon sources

Assays were done in potassium phosphate buffer (130 mM, pH 8.0) using the direct assay. ND, Not done.

Assay substrate* (80 μ M)	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹] after growth on:		
	Succinate	Dodecanediol	Hexadecanol
C ₁₆ -SCoA	27.7	35.4	29.4
16HO C ₁₆ -SCoA	44.4	55.6	46.5
DC ₁₆ -SCoA ₁	10.2	5.9	7.1
DC ₁₆ -SCoA ₂	ND	ND	1.5
C ₁₂ -SCoA	38.6	29.5	22.3
12HO C ₁₂ -SCoA	5.2	3.8	4.0
DC ₁₂ -SCoA ₁	3.2	1.5	2.3
DC ₁₂ -SCoA ₂	ND	ND	0

* C₁₆-SCoA, palmitoyl-CoA; 16HO C₁₆-SCoA, 16-hydroxypalmitoyl-CoA; DC₁₆-SCoA₁, mono CoA ester of hexadecanedioic acid; DC₁₆-SCoA₂, di CoA ester of hexadecanedioic acid; C₁₂-SCoA, lauroyl-CoA; 12HO C₁₂-SCoA, 12-hydroxylauroyl-CoA; DC₁₂-SCoA₁, mono CoA ester of dodecanedioic acid; DC₁₂-SCoA₂, di CoA ester of dodecanedioic acid.

on fatty acids (Klein *et al.*, 1971; Overath *et al.*, 1967, 1969; Weeks *et al.*, 1969) and is subject to catabolite repression by glucose (Weeks *et al.*, 1969).

The β -oxidation system of *Corynebacterium* sp. strain 7E1C displayed a marked preference for long-chain monocarboxylic acids (Fig. 1). Dicarboxylic acids, (DC₁₀, DC₁₂, DC₁₄ and DC₁₆) were relatively very poor substrates in both the linked assay (free fatty acids as substrates; Table 3) and in the direct assay (acyl-CoA esters as substrates; Table 4). For ω -hydroxycarboxylic acids as substrates, a dramatic change was observed on

Table 5. Activities present in crude cell-free extracts of hexadecanol-grown *Corynebacterium* 7E1C which interfere with the measurement of β -oxidation

All activities assayed in potassium phosphate buffer (130 mM, pH 8.0).

Enzyme	Substrate	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]
β -Oxidation*	Lauroyl-CoA (80 μ M)	37.4
	12-Hydroxylauroyl-CoA (80 μ M)	4.1
	16-Hydroxypalmitoyl-CoA (80 μ M)	31.7
Thioesterase	Acetyl-CoA (80 μ M)	10.6
	Acetyl-CoA (37 μ M)	4.1
	Lauroyl-CoA (80 μ M)	43.0
ω -Hydroxyfatty acid dehydrogenase	12-Hydroxylaurate (80 μ M)	0.8
	16-Hydroxypalmitate (80 μ M)	0
'NADH oxidase'	NADH (37 μ M)	17.1
	NADH (18.5 μ M)	14.6

* Measured as rate of NAD⁺ reduction.

increasing chain-length: 12-hydroxylaurate was a poor substrate, comparable to the dicarboxylic acids, but 16-hydroxypalmitate was a good one (Tables 3 and 4).

It is noteworthy that the cell-free extracts contain a number of activities that could possibly compromise estimates of β -oxidation activity. These are NADH oxidase, long-chain acyl-CoA thioesterase and ω -hydroxy fatty acid dehydrogenase (Table 5). The thioesterase and NADH oxidase activities would tend to lead to an underestimation of β -oxidation activity; the thioesterase had the effect of rapidly depleting the substrate concentration so that assays were linear for only a short time (3–4 min). However, as initial rates of β -oxidation were determined and as the effects of these contaminating activities would be time-dependent, errors associated with them are probably quite small. As indicated above, the alcohol dehydrogenase activity did not affect the rates of β -oxidation with the ω -hydroxy fatty acids.

Acyl-CoA synthetase

Measurements of acyl-CoA synthetase activity with laurate as substrate showed the expected cofactor requirements. On omission of fatty acid (laurate) or using boiled extract, no activity was observed. On omission of ATP, the activity fell to 13% of that seen with the complete mixture. The residual activity was probably due to carry over of ATP from the extract. ATP could not be replaced by GTP, CTP, UTP or TTP (data not shown).

Acyl-CoA synthase activity was also constitutive: both lauroyl-CoA synthetase [25 ± 5 nmol min⁻¹ (mg protein)⁻¹] and DC₁₂-CoA synthetase [17 ± 7 nmol min⁻¹ (mg protein)⁻¹] activities were expressed similarly after

growth on either succinate, 1,12-dodecanediol, hexadecanol, hexadecane or glucose. Again, this is in marked contrast to the situation in *E. coli* (Overath *et al.*, 1969; Samuel & Ailhand, 1969; Weeks *et al.*, 1969). It is also in contrast to the inducible dicarboxyl-CoA synthetase of *Acinetobacter calcoaceticus* (Modrzakowski & Finnerty, 1989).

The acyl-CoA synthetase in crude extracts of *Corynebacterium* sp. strain 7E1C displayed a very wide substrate specificity (Fig. 2). An essentially identical substrate specificity was observed with extracts prepared from cells grown on succinate, 1,12-dodecanediol or hexadecanol. For any given carbon source (for growth), the rate of activation of long-chain (C₁₀–C₁₆) dicarboxylic acids was in the range of 50 to 100% of the rate for the corresponding monocarboxylic acid. Kinetic studies under the conditions described in Fig. 2 confirmed that long-chain carboxylic, ω -hydroxycarboxylic and dicarboxylic acids were good substrates. Approximate K_m values of < 100 μ M were obtained for decanoic, dodecanoic and tetradecanoic acids. For palmitate, the value was 150 μ M.

Alcohol dehydrogenases

Soluble NAD⁺- and NADP⁺-dependent alcohol dehydrogenases were detected in cell extracts of *Corynebacterium* sp. strain 7E1C. The activities were similar after growth on a wide range of carbon sources, but NADP⁺-octanol dehydrogenase activity was stimulated by growth on alkyl-group-containing substrates (Broadway, 1990). No nucleotide-independent alcohol dehydrogenase was detected. The alcohol dehydrogenases are stimulated 3- to 4-fold by high salt concentrations and assays were routinely performed in either potassium phosphate buffer

Fig. 2. Substrate specificity of acyl-CoA synthetase activity in extracts of hexadecanol-grown *Corynebacterium* 7E1C. Assays were performed in potassium phosphate buffer, pH 8.0, as described in the text. 100% [refers to the activity observed with lauric acid (C_{12})] = 20 nmol min⁻¹ (mg protein)⁻¹. ▨, Monocarboxylic acid; ■, ω-hydroxycarboxylic acid (C_4 , C_6 , C_8 and C_{14} ω-hydroxymonocarboxylic acids were not tested); □, dicarboxylic acid.

Table 6. Summary of the kinetic parameters of NAD⁺-dependent ω-hydroxyfatty acid dehydrogenase activity in a crude cell-free extract of hexadecanol-grown *Corynebacterium* 7E1C

Assayed in glycine buffer (50 mM, pH 10) containing 0.8 M- Na_2SO_4 .

Substrate	K_m (μM)	V_{max} [nmol min ⁻¹ (mg protein) ⁻¹]
10-Hydroxydecanoate	1520	50
12-Hydroxylaurate	77	44
16-Hydroxypalmitate	22	42

(0.8 M, pH 8.0) or glycine buffer (50 mM, pH 10.0) containing 0.8 M- Na_2SO_4 . Substrate specificity studies showed that with both buffers the optimal substrate for NAD⁺- and NADP⁺-linked alcohol dehydrogenase was octanol, whilst 1,12-dodecanediol was the optimum α, ω-diols. Importantly, 1,16-hexadecanediol was a poor substrate. It was difficult to determine the specificity of the enzyme towards ω-hydroxy acids in the potassium phosphate buffer system due to the combination of low rates, unfavourable equilibrium constant and high K_m

for the ω-hydroxy fatty acid. Conditions were more favourable at pH 10.0 and the results obtained are recorded in Table 6.

Discussion

Only a very narrow range of dicarboxylic acids was produced by *Corynebacterium* sp. strain 7E1C (Table 1) or its derived DC-accumulating mutant, *Corynebacterium* sp. ATCC 21747, after growth on alkanes or fatty acid esters. Whenever a dicarboxylic acid product was observed, the principal product had the same chain-length (C_{12} , C_{14}) as the growth substrate. This suggests that the product arises by direct diterminal oxidation of the alkane substrate (Fig. 3), and not as a result of ω-oxidation of chain-shortened fatty acids arising from β-oxidation. If the latter did occur, one might expect significant DC₁₂ production from cells grown on hexadecane and this does not happen (Table 1). The

Fig. 3. Pathways of dicarboxylic acid formation from alkanes. 1, Hydroxylase; 2, alcohol dehydrogenase; 3, aldehyde dehydrogenase. [], Compound has never been isolated.

dicarboxylic acids that are formed are not peculiarly inert as substrates for β -oxidation when they are compared to other dicarboxylic acids (Table 2); this process probably accounts for the small amounts of chain-shortened dicarboxylic acids found (Table 1). However, long-chain dicarboxylic acids are poor substrates for β -oxidation as compared to fatty acids of the same chain-length (Tables 3 and 4). This is not because of the specificities of fatty acyl-CoA synthetases (or indeed the long-chain acyl-CoA thioesterases), which do not discriminate greatly between mono- or dicarboxylic acids (or their thio esters) (Broadway, 1990). It is probably due to the specificity of the long-chain fatty acyl-CoA dehydrogenase, which is the first enzyme of the β -oxidation sequence. This enzyme appears to be rate-limiting for oxidation of both mono- and dicarboxylic acids alike (Broadway *et al.*, 1992).

If fatty acids and dicarboxylic acids arise by terminal oxidation of alkanes and fatty acids, as shown in Fig. 3, the relative amounts formed will clearly depend on the specificities of the hydroxylase and alcohol and aldehyde dehydrogenase enzymes. If the specificities are such that a greater proportion of dodecane is converted to DC₁₂ than hexadecane is to DC₁₆ then, because of the slow β -oxidation of the dicarboxylic acids, DC₁₂ would be more likely to accumulate. We have not made any studies of the hydroxylase or aldehyde dehydrogenase and the characterization of alcohol dehydrogenase and β -oxidation is only partial. However, the work suggests that if ω -hydroxy fatty acids are significant metabolites in dicarboxylic acid formation and, as shown in Fig. 3 they are thought to be obligatory intermediates, then the kinetic characteristics of alcohol dehydrogenase and β -oxidation (Tables 3, 4 and 6) would favour dicarboxylic acid formation from 12-hydroxylaurate and β -oxidation for 16-hydroxypalmitate. The degree of discrimination achieved would clearly depend on the precise conditions obtaining in the cells. However, the observation that no DC₁₆ could be detected in cultures of cells grown on 16-hydroxypalmitate (here the problem of ω -hydroxylase specificity is circumvented) demonstrates that, under these conditions at least, β -oxidation of 16-hydroxypalmitate is favoured over ω -oxidation to DC₁₆. It is important to emphasize that for there to be no accumulation of DC₁₆ it is not necessary for there to be no conversion of 16-hydroxypalmitate to DC₁₆. As long as the rate of DC₁₆ production and degradation are balanced, there will be no accumulation. Although the rate of β -oxidation of DC₁₆ is low it is still significant.

The suggestions presented above illustrate the arguments about the specificities of the enzymes in Fig. 3, but it is likely that the hydroxylase as the rate-limiting step in Fig. 3 is the decisive step in determining the relative rates of mono- and dicarboxylic acid production from alkane

or fatty acid. More work here is clearly needed. Also, whilst the specificities of the enzymes in Fig. 3 are important, other factors may also contribute. The cells used here are orange-red in colour and it was quite evident that the bacteria grew less well as the chain-length of the substrate was reduced to C₁₂ and below. In fact with methyl laurate, there was some conversion of substrate to DC₁₂ but no obvious growth (Table 1). With less vigorous growth, the decreased demand for energy would lead to lower rates of β -oxidation of fatty acids. These, being used less rapidly, are more likely to undergo terminal hydroxylation and would be more likely to be converted into dicarboxylic acids.

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