Long-chain acyl-CoA ester intermediates of β -oxidation of mono- and di-carboxylic fatty acids by extracts of *Corynebacterium* sp. strain 7E1C

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β -Oxidation of palmitate and tetradecanedioic acid was studied in cell-free extracts of the Gram-positive bacterium *Corynebacterium* sp. strain 7E1C, and the acyl-CoA ester intermediates formed were analysed by h.p.l.c. β -Oxidation assays displayed a lag phase before a constant rate of NAD⁺ reduction was obtained. The length of the lag phase was inversely proportional to the number of units of activity added to assays. This is a characteristic feature of a system of consecutive reactions proceeding via free intermediates. During β -oxidation of palmitate all the saturated acyl-CoAs from C₁₆ to C₈ were detected together with trace amounts of unsaturated and 3-hydroxy-intermediates. The time-course of intermediate formation again indicated a precursor–product relationship indicative of free intermediates being formed. When 3-hydroxyacyl-CoA dehydrogenase was inhibited by completely removing NAD⁺ from assays, the major acyl-CoAs, detected during palmitate β -oxidation were palmitoyl-CoA, hexadeca-2-enoyl-CoA and 3-hydroxypalmitoyl-CoA. These compounds also displayed a precursor–product relationship. Under normal assay conditions the acyl-CoA dehydrogenase(s) are the probable rate-limiting enzyme(s) of the β -oxidation spiral. These results indicate that in cell-free extracts of *Corynebacterium* sp. strain 7E1C, β -oxidation proceeds via free acyl-CoA intermediates and is at variance with the concept of substrate channelling or of a ‘leaky hose pipe’ model as proposed for mitochondrial β -oxidation in eukaryotic cells. The significant accumulation of chain-shortened acyl-CoA esters is similar to the situation observed for mammalian peroxisomal β -oxidation.

INTRODUCTION

It was long thought that the β -oxidation of fatty acids proceeds without the formation of free intermediates and that once a fatty acid enters the β -oxidation spiral it is completely degraded to acetyl-CoA without release from the β -oxidation enzymes (see Greville & Tubbs, 1968). Garland *et al.* (1965) found that in intact mitochondria oxidizing palmitate, virtually all the intra-mitochondrial CoA could be accounted for as acetyl-CoA, long-chain (palmitoyl-) acyl-CoA or as free CoASH. There were no significant concentrations of chain-shortened acyl-CoA intermediates. The lack of β -oxidation intermediates prompted the suggestion that the mitochondrial β -oxidation enzymes are organized in the form of a multienzyme complex (Greville & Tubbs, 1968). Since then Stanley & Tubbs (1974, 1975) detected C₁₄ through to C₈ saturated intermediates during the β -oxidation of palmitate by intact mitochondria. However, they demonstrated that these intermediates were not on the main pathway and proposed a ‘leaky hose pipe’ model of β -oxidation in which the intermediates represented leakage. Similar results were obtained by Watmough *et al.* (1989) who detected only myristoyl-CoA (C₁₄) and traces of lauryl-CoA (C₁₂) during mitochondrial β -oxidation of palmitate (C₁₆). This lack of intermediates is consistent with some form of structural organization of the β -oxidation enzyme with resultant substrate channelling. Bartlett *et al.* (1990) demonstrated the presence of all the saturated acyl-CoA intermediates from C₁₆ down to C₂ during the peroxisomal β -oxidation of palmitate. Thus the behaviour of the peroxisomal and mitochondrial β -oxidation systems is rather different with respect to intermediate accumulation.

During investigations of β -oxidation by cell-free extracts of *Corynebacterium* sp. strain 7E1C using a linked assay in which

acyl-CoAs were generated *in situ*, it became apparent that there was a significant accumulation of long-chain acyl-CoA. The nature of the accumulated acyl-CoA was investigated with a view to determining the behaviour of the bacterial β -oxidation system and how this compares with the mitochondrial and peroxisomal systems of higher organisms.

MATERIALS AND METHODS

Corynebacterium sp. strain 7E1C (ATCC 19067) was used in all experiments. CoA (trilithium salt), acyl-CoA esters, ATP (type II), citrate synthase, DNAase I, acyl-CoA oxidase (*Candida* sp.) and crotonase were purchased from Sigma Chemical Co., Poole, Dorset, U.K. NAD⁺ (grade II) was obtained from Boehringer-Mannheim U.K., Lewes, East Sussex, U.K. Palmitate and tetradecanedioic acid were from Aldrich Chemical Co., Gillingham, Dorset, U.K. All other reagents were of the highest grade commercially available.

β -Oxidation assays

β -Oxidation was assayed at 25 °C by following NAD⁺ reduction by the 3-hydroxyacyl-CoA dehydrogenase or by measuring acetyl-CoA production essentially as described by Kawamoto *et al.* (1978).

Linked assay

In this assay the acyl-CoA esters were generated *in situ* by the acyl-CoA synthetases present in the extract. The assay was performed in a final volume of 0.9 ml with additions at the following final concentrations: KH₂PO₄/K₂HPO₄ buffer, pH 8.0 (120 mM); ATP (disodium salt) (3.6 mM); CoASH (trilithium salt) (72 μ M); MgCl₂·6H₂O (3.6 mM); NAD⁺ (1.7 mM); cell-free extracts; fatty acid substrate (0.68 μ mol, introduced

Abbreviations used: DC_n, α,ω -dicarboxylic acid with *n* carbon atoms; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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Table 1. Cofactor requirements of linked assays of β -oxidation in crude extracts of succinate-grown *Corynebacterium* sp. strain 7E1C

Assays contained 0.75 mM-sodium laurate and 0.1 mg of protein.
100% = 30 nmol/min per mg of protein. n.d., not determined.

Component omitted	Relative activity (%)	
	NAD ⁺ reduction	Acetyl-CoA formation
None	100	100
ATP	13	6
CoASH	2	2
MgCl ₂	80	n.d.
NAD ⁺	24*	24
NADP ⁺ replaced NAD ⁺	3	n.d.

* Rate fell to zero within 2 min.

Fig. 1. Progress curves observed in assays of β -oxidation in succinate-grown *Corynebacterium* sp. strain 7E1C

The assay was conducted at 25 °C and pH 8.0 as described in the text. The formation of NADH in the reactions was followed spectrophotometrically at 340 nm. The reaction was initiated by addition of sodium laurate. τ is the transition time referred to in the text. Curve A shows a linked assay and curve B a direct assay. Extract (20 μ l; 0.1 mg of protein) was added to the 0.9 ml assays. It should be noted that the observed steady-state rate was always somewhat greater in the direct assay.

into the assay in 7 μ l of dimethyl sulphoxide). The reaction was initiated by substrate addition. Activity was routinely followed by measuring NAD⁺ reduction at 340 nm (ϵ = 6220 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹). Activity could also be followed by measuring acetyl-CoA production.

Direct (acyl-CoA-dependent) assay

The assay was performed in a final volume of 0.9 ml and contained the following components: KH₂PO₄/K₂HPO₄ buffer, pH 8.0 (120 mM), NAD⁺ (1.7 mM), cell-free extract and acyl-CoA ester (65 nmol, introduced as an aqueous solution). The reaction was initiated by acyl-CoA addition.

Measurement of acetyl-CoA production in β -oxidation assays

Acetyl-CoA was assayed using citrate synthase in the presence of oxaloacetate. CoASH released on condensation of acetyl-CoA with oxaloacetate was quantified by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). A sample (200 μ l) of reaction mixture was mixed with trichloroacetic acid (60 μ l, 15%, w/v) and protein precipitated by centrifugation. A portion (200 μ l) of supernatant was added to Tris/HCl buffer, pH 8.0 (0.8 M; 0.8 ml) containing excess oxaloacetate (approx. 2 mg solid) and DTNB (250 nmol). The absorbance at 412 nm (A_1) was read against a

reagent blank. Citrate synthase (1 unit) was added and the increased absorbance at 412 nm (A_2) due to the release of CoASH from acetyl-CoA measured. ϵ_{412} 13600 l $\cdot$ mol⁻¹ $\cdot$ cm⁻¹.

In the linked assay total acyl-CoA formation could be determined by measuring total CoASH consumption, i.e.

$$A_1(t=0) - A_1(t=x) = \text{total acyl-CoA at } t=x$$

$$A_2(t=x) - A_1(t=x) = \text{acetyl-CoA at } t=x$$

'Long-chain' acyl-CoA = total acyl-CoA - acetyl-CoA. Note: 'long-chain' refers to acyl-CoAs other than acetyl-CoA.

Synthesis of acyl-CoA esters

The mono-CoA ester of tetradecanedioic acid (DC₁₄) was synthesized via the acyl-imidazole method (Kawaguchi *et al.*, 1981) and purified by h.p.l.c. using the same protocol as for acyl-CoA analysis (see below). *trans*-Hexadec-2-enoyl-CoA was synthesized from palmitoyl-CoA using acyl-CoA oxidase from *Candida* sp. (Sigma). The reaction mixture (1 ml) contained 100 μ mol of KH₂PO₄/K₂HPO₄ buffer, pH 7.5, horseradish peroxidase (0.1 unit), acyl-CoA oxidase (0.1 unit), 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (0.8 μ mol) and palmitoyl-CoA (80 nmol). Reaction was initiated by the addition of acyl-CoA. When ΔA_{405} corresponding to the oxidation of 40 nmol of acyl-CoA was recorded the reaction was quenched by the addition of acetic acid (200 μ l). Acyl-CoA esters were extracted and purified as described below. 3-Hydroxyacyl-CoA esters were synthesized as described above except that crotonase (0.1 unit) was added to the reaction mixture.

H.p.l.c. analysis of acyl-CoA esters

H.p.l.c. was performed essentially as described by Causey & Bartlett (1986) with minor modifications. A Kontron 420 h.p.l.c. pump and Kontron 425 h.p.l.c. gradient former were used with a reversed-phase Lichrosorb 10 μ RP C₁₈ column (250 mm $\times$ 4.6 mm) held at 30 °C. Column eluates were monitored by measuring A_{260} . Peak heights and areas were measured by a Kontron I-459 integrator. The following gradient of acetonitrile in 50 mM-KH₂PO₄ (pH 5.3) was used: 5% (v/v) acetonitrile for 5 min; then a linear increase to 40% (v/v) acetonitrile (7.5 min); followed by a linear increase to 50% (v/v) acetonitrile (5 min). The flow rate was 1 ml/min. The mobile phases were continuously sparged with helium. Sample preparation was performed by a modification of the method of Causey & Bartlett (1986). A portion (0.5 ml) from a β -oxidation assay was quenched with acetic acid (100 μ l). An acyl-CoA internal standard (50 nmol) was added and the reaction mixture extracted with 3 $\times$ 5 ml of diethyl ether to remove fatty acids. Saturated (NH₄)₂SO₄ (50 μ l) was added to the aqueous phase. Methanol/chloroform (2:1, v/v; 3 ml) was added slowly and the mixture left for 20 min at room temperature. The salt-protein precipitate was sedimented by centrifugation (1000 g, 5 min) and the supernatant removed. The salt-protein pellet was extracted with a further 3 ml of chloroform/methanol (2:1, v/v) and the supernatants were combined. The solvent was removed under a N₂ stream at 55 °C. The residue was redissolved in 200 μ l of 50 mM-KH₂PO₄ (pH 5.3) and a sample analysed by h.p.l.c.

Growth of bacterium

A loopful of bacteria was transferred from a nutrient broth slope to nutrient broth (100 ml) in a 250 ml conical flask and grown for 24–36 h at 30 °C on a rotary shaker at 160 rev./min. A sample (10 ml) was then transferred to Jays medium (see below) (800 ml) containing succinate (1%, w/v) or hexadecanol (0.2%, w/v) in a 1-litre vortex-aerated bottle. Cultures were held at 30 °C and cells were harvested during mid- to late-exponential

Fig. 2. Acetyl-CoA and acyl-CoA formation in the linked assay of palmitate by extracts of succinate-grown *Corynebacterium* sp. strain 7E1C

Assays were performed at 25 °C and pH 8.0 in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer as described in the text. The incubation mixture (2.7 ml) contained 0.1 mg of crude extract protein. ○, Acyl-CoA; ▲, acetyl-CoA. This experiment was repeated on three other occasions with almost identical results.

growth phase. Jays medium is essentially the same as that described by Jayasuria (1955), except that the phosphate buffer concentration is doubled. The medium contained, per litre of distilled water: KH_2PO_4 , 2.72 g; Na_2HPO_4 , 4.26 g; $(\text{NH}_4)_2\text{SO}_4$, 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g; MnSO_4 , 0.0025 g; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.0025 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g; yeast extract 1.0 g; pH 7.0. All media were sterilized by autoclaving at 121 °C for 15 min. Cells were harvested by centrifugation at 25000 g for 15 min at 4 °C. Cells were washed three times in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (100 mM, pH 7.5) and the final pellet was resuspended in the minimum volume of Hepes buffer (20 mM, pH 7.5). Cells were disrupted by three passages through a French pressure cell (35 MPa). After the first passage, DNAse I (approx. 2 mg) was added to the extract which was highly viscous due to the presence of DNA. Whole cells and cell debris were removed by centrifugation at 8000 g for 15 min at 4 °C. The supernatant fraction was designated 'crude cell-free extract' and was either used immediately or stored in small

portions in liquid N_2 until required. Extracts were stable indefinitely when stored in this manner although repeated freeze-thawing resulted in significant loss of β -oxidation activity.

RESULTS AND DISCUSSION

β -Oxidation assays

The cofactor requirements of linked assays were those expected (Table 1). Direct assays were independent of both ATP and CoASH, the only requirement being for acyl-CoA (which could not be replaced by free fatty acid) and NAD^+ . In direct assays, endogenous activity (i.e. the rate in the absence of added fatty acid) was essentially zero regardless of the growth substrate for the cells. With the linked assay, on the other hand, endogenous activity was very high when cells were grown on long-chain substrates (hexadecan-1-ol), but very low in extracts from cells grown on succinate. As a result, the linked assay could only be used for extracts from succinate-grown cells.

Cell free extracts were found to contain NADH oxidase, acetyl-CoA thioesterase and long-chain acyl-CoA thioesterase. The acetyl-CoA thioesterase and NADH oxidase would tend to result in under estimation of the activity of the β -oxidation system as measured by acetyl-CoA and NADH production. The long-chain thioesterase activity had the effect of rapidly decreasing the acyl-CoA substrate concentration so that assays were only linear for 3–4 min. The rather small effect seen in Table 1 on omission of MgCl_2 is a little surprising, but the same effect (75% activity) was seen in a second experiment. It seems most likely that the effect was due to carry over of Mg^{2+} from the crude extract. The K_m for ATP under the conditions of the experiment was of the order of 0.1 mM so that little Mg^{2+} was required to support strong activity.

β -Oxidation assays displayed a lag phase during which the rate of NAD^+ reduction (or acetyl-CoA production) increased with time until a constant linear rate was obtained (Fig. 1). The lag was more pronounced in the linked than in the direct assay (see the Materials and methods section). The length of the lag (or transition time, τ) was inversely proportional to the amount of

Fig. 3. H.p.l.c. analysis of acyl-CoA intermediates formed by β -oxidation in the 'linked' β -oxidation assay of extracts of succinate-grown *Corynebacterium* sp. strain 7E1C

The β -oxidation assay was performed at 25 °C and pH 8.0 in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer as described in the text. The acyl-CoA esters were separated on a 10 μl Lichrosorb RP C_{18} column. Samples were taken after 60 min incubation. The protein concentration in incubation mixtures was 0.17 mg/ml. (a) Intermediates formed in the oxidation of palmitate: A, butyryl-CoA; B, hexanoyl-CoA; C, octanoyl-CoA; D, decanoyl-CoA; E, lauroyl-CoA; F, myristoyl-CoA; G, palmitoyl-CoA; IS, internal standard (pentadecanoyl-CoA; 50 μM). (b) Intermediates formed in the oxidation of 1,12-dodecanedioic acid. A, mono-CoA ester of 1,12-dodecanedioic acid; B, di-CoA-esters of 1,12-dodecanedioic acid; C, lauroyl-CoA (internal standard; 100 μM). (c) Intermediates formed in the oxidation of palmitate when 3-hydroxyacyl-CoA dehydrogenase is inhibited. A, butyryl-CoA; B, hexanoyl-CoA; C, octanoyl-CoA; D, decanoyl-CoA; E, lauroyl-CoA; F, myristoyl-CoA; G, palmitoyl-CoA; Y, tetradecenoyl-CoA/3-hydroxypalmitoyl-CoA; Z, hexadec-2-enoyl-CoA; IS, internal standard (pentadecanoyl-CoA; 50 μM).

Fig. 4. Time-course of acyl-CoA intermediate formation during β -oxidation by extracts of succinate-grown *Corynebacterium* sp. strain 7E1C

Assays were performed at 25 °C in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer at pH 8.0. Each experiment was only performed in full once. However, in each case an additional experiment was conducted in which only two or three samples were taken for analysis. The results of these limited experiments supported the findings of the more detailed studies in that long-chain acyl-CoA esters appeared early on and the short-chain esters appeared only after prolonged incubation when the concentrations of long-chain esters were declining. The assays shown contained crude extract protein at concentrations of (a) 0.14 mg/ml, (b) 0.17 mg/ml and (c) 0.17 mg/ml. (a) Assay of the oxidation of 80 μM -palmitoyl-CoA by the 'direct' method (see the text). Acyl-CoA esters were determined by h.p.l.c. $\circ$, Palmitoyl-CoA; $\bullet$, myristoyl-CoA; $\triangle$, lauroyl-CoA; $\blacktriangle$, decanoyl-CoA. 100% = 80 μM -acyl-CoA. (b) Assay of the oxidation of 0.75 mM-palmitate by the 'linked' method (see the text). Acyl-CoA esters were determined by h.p.l.c. $\circ$, Palmitoyl-CoA; $\bullet$, myristoyl-CoA; $\triangle$, lauroyl-CoA; $\blacktriangle$, decanoyl-CoA; $\square$, octanoyl-CoA. 100% = 50 μM -acyl-CoA. (c) Assay of oxidation of 0.75 mM-palmitate by the 'linked' method with 3-hydroxyacyl-CoA dehydrogenase inhibited (see the text). Acetyl-CoA was measured with citrate synthase, and other acyl-CoA esters were determined by h.p.l.c. $\circ$, Palmitoyl-CoA; $\triangle$, *trans*- Δ^2 -hexadecenoyl-CoA; $\bullet$, 3-hydroxypalmitoyl-CoA; $\blacksquare$, acetyl-CoA. 100% = 50 μM -acyl-CoA.

extract added in both the linked and the direct assays. This is the expected behaviour for a series of consecutive reactions involving free intermediates in which the concentration of the product of the first reaction must build up to its steady-state concentration before the next reaction can proceed maximally and so on down the series of reactions (Easterby, 1981; Kuchel, 1985). The lag observed when β -oxidation in *Corynebacterium* sp. strain 7E1C was assayed implies a pool of free intermediates of the reaction pathway (Easterby, 1981; Kuchel, 1985). The implications of this behaviour with respect to the structural organization of β -oxidation in this organism are discussed below.

Intermediates of β -oxidation

The β -oxidation of palmitate was investigated using both the linked and the direct assays. With both assays, samples were removed periodically and worked-up for h.p.l.c. analysis of the acyl-CoA esters present. Additionally, with the linked assay, duplicate samples were removed to determine the total CoASH consumption and acetyl-CoA production. Hence, by difference, the total long-chain acyl-CoA could be determined (see the Materials and methods section). It then became apparent that there was a significant accumulation of long-chain acyl-CoA

(Fig. 2) of unknown identity. The nature of this accumulated long-chain acyl-CoA was investigated by h.p.l.c. (Bartlett *et al.*, 1988; Causey & Bartlett, 1986; Watmough *et al.*, 1989).

A typical h.p.l.c. trace, obtained with samples taken from a linked assay after 60 min incubation, shows the presence of all saturated acyl-CoA from C_8 to C_{16} (Fig. 3). In the direct assay, all saturated acyl-CoAs from C_{10} to C_{16} were detected (trace not shown). In a control incubation lacking fatty acid, no acyl-CoA esters could be detected.

It is important to note that the major acyl-CoA of each chain length is the saturated compound. In some cases small amounts of what may be the Δ^2 -enoyl- (retention time slightly shorter than the corresponding saturated compound) and/or the 3-hydroxyacyl-CoA (retention time slightly shorter than the C_{n-2} saturated compound) (Watmough *et al.*, 1989) were detected. However, these peaks are extremely small compared with the saturated acyl-CoA peaks. This indicates that for C_8 to C_{16} chain lengths the acyl-CoA dehydrogenase(s) is (are) the rate-limiting step in the β -oxidation spiral.

The time-course of the production of the various-chain-length acyl-CoAs is shown in Fig. 4(a,b). These time-courses indicate a precursor-product relationship between the different-chain-

Fig. 5. Time course of acetyl-CoA and total acyl-CoA formation during β -oxidation of palmitate in the 'linked' β -oxidation assay

Assays were performed at 25 °C and pH 8.0 in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer. The protein concentration was 0.17 mg/ml. Δ , Acetyl-CoA; $\circ$, long-chain acyl-CoA (measured as total CoASH consumed – acetyl-CoA); $\bullet$, total acyl-CoA as determined by h.p.l.c.

length acyl-CoA esters. Each acyl-CoA, in turn, builds up to its maximum concentration before decreasing as the concentration of the C_{n-2} acyl-CoA builds up to its own maximum concentration before in turn decreasing and so on down the homologous series. This is the behaviour predicted for a series of consecutive linked reactions involving free intermediates (Stewart *et al.*, 1973; Easterby, 1981; Kuchel, 1985). By 'free intermediates' it is meant that intermediates are not so tightly bound to β -oxidation enzymes as not to be in equilibrium with the bulk aqueous phase.

The time-course of long-chain acyl-CoA formation determined enzymically and that of long-chain acyl-CoA determined by h.p.l.c. (i.e. the sum of the acyl-CoAs of different chain lengths) are essentially identical (Fig. 5).

β -Oxidation of DC_{14} was investigated using the linked assay. The main acyl-CoA detected by h.p.l.c. was the mono-CoA ester of DC_{14} although on prolonged incubation small amounts of DC_{12} mono-CoA ester were also detected. The experiment was repeated once with essentially the same result. The low concentration of chain-shortened intermediates detected during β -oxidation of DC_{14} can be accounted for by the observation that DC_{14} is a poor β -oxidation substrate (oxidized at approx. 15% the rate of palmitate), and only limited β -oxidation of DC_{14} was observed in this experiment. Pourjarzam & Bartlett (1991) reported the β -oxidation of DC_{16} -CoA by rat liver mitochondria and peroxisomes. In peroxisomes DC_{16} -CoA yielded the C_4 – C_6 saturated intermediates whereas in mitochondria no intermediates were seen. It is important to note that the only chain-shortened acyl-CoA esters detected are saturated ones, implying that the acyl-CoA dehydrogenase(s) is(are) rate-limiting in the β -oxidation of dicarboxylic acids.

Effect of inhibition of 3-hydroxyacyl-CoA dehydrogenase on intermediate formation

The effects of 3-hydroxyacyl-CoA dehydrogenase inhibition on intermediate formation were investigated using palmitate in the linked assay. The enzyme was inhibited by pre-incubating the cell-free extract (1 ml, approx. 3 mg/ml protein) for 5 min at room temperature with NADase (0.1 unit) and omitting NAD^+ from the assay. The pattern of intermediate accumulation (Fig. 4c) is very different from that observed under conditions when 3-hydroxyacyl-CoA dehydrogenase is fully active. Three major acyl-CoA species were now detected; palmitoyl-CoA, hexadec-2-

enoyl-CoA and 3-hydroxypalmitoyl-CoA. Small amounts of decanoyl- and lauroyl-CoA esters were also detected along with what appeared to be their Δ^2 -enoyl- and 3-hydroxy- derivatives. No acetyl-CoA production could be detected when assayed enzymically with citrate synthase which indicates that 3-hydroxyacyl-CoA dehydrogenase was severely inhibited. However, 3-hydroxyacyl-CoA dehydrogenase may still have been able to turn over extremely slowly which would explain the small amounts of chain-shortened acyl-CoA detected. The time-course of intermediate formation is shown in Fig. 4(c). The detection of Δ^2 -unsaturated and 3-hydroxyacyl-CoA esters under these conditions demonstrates that the inability to detect these compounds under normal conditions is not due to the analytical system failing to resolve them from saturated acyl-CoAs. These results are in agreement with those obtained with the mammalian liver mitochondrial β -oxidation system where 3-hydroxyacyl-CoA esters accumulate significantly only when NADH dehydrogenase is inhibited by either rotenone (Bremer & Wojtczak, 1972; Stanley & Tubbs, 1974, 1975; Watmough *et al.*, 1989) or high NADH/ NAD^+ ratio (Lopes-Cardozo *et al.*, 1978). Similarly Bartlett *et al.* (1990) have detected unsaturated intermediates during peroxisomal β -oxidation of palmitate only in the absence of an NAD^+ -regenerating system.

Significance of the accumulation of β -oxidation intermediates

The accumulation of β -oxidation intermediates by *Corynebacterium* sp. strain 7E1C contrasts markedly with observations made for mammalian mitochondrial β -oxidation and is more akin to the peroxisomal system. However, although significant accumulation of saturated acyl-CoA esters has been demonstrated during peroxisomal β -oxidation (Bartlett *et al.*, 1990), the results presented by these authors did not indicate an obvious precursor-product relationship between the different-chain-length acyl-CoAs. The results presented here, however, indicate that the functional organization of β -oxidation in *Corynebacterium* sp. strain 7E1C is significantly different from that of mammalian mitochondria. The lag observed in both the linked and direct assays, before a maximum linear rate of β -oxidation is obtained, is consistent with β -oxidation proceeding via free intermediates. The detection of C_8 – C_{10} intermediates during β -oxidation of palmitate is compelling evidence for β -oxidation proceeding via free intermediates. Furthermore, the time-course of appearance and disappearance of the various-chain-length acyl-CoA esters indicates a precursor-product relationship and is therefore at variance with a 'leaky hose pipe' model of β -oxidation.

The bacterial β -oxidation system, as exemplified by *Escherichia coli*, displays a remarkable degree of structural organization. It consists of two subunits. One subunit possesses the combined activities of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase and enoyl-CoA isomerase (see Nunn, 1986), whereas the other subunit possesses only 3-oxoacyl-CoA thiolase activity. Such a system should be highly efficient in catalysing the β -oxidation sequence of reactions. Indeed, it has been demonstrated (Yang *et al.*, 1986) that dec-2-enoyl-CoA is converted into 3-oxodecanoyl-CoA without a lag in product (NADH) formation by this complex. Although the mammalian peroxisomal bifunctional protein appeared to function in a similar way, when the *E. coli* complex was replaced with purified mitochondrial enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, a definite lag in NADH production was observed. The difference observed in the two situations was explained by substrate channelling in the *E. coli* β -oxidation complex.

The behaviour of the β -oxidation systems of *E. coli* and *Corynebacterium* sp. strain 7E1C appear to be significantly

different. However, with *Corynebacterium* sp. strain 7E1C in the present work, acyl-CoA was used as substrate as opposed to the enoyl-CoA in the *E. coli* experiments. The *E. coli* acyl-CoA dehydrogenase is not associated with the β -oxidation complex and, although some substrate channelling occurs between enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase sites on the multifunction protein, such channelling may not occur between acyl-CoA dehydrogenase and enoyl-CoA hydratase or between thiolase and acyl-CoA dehydrogenase at the end of each turn of the β -oxidation spiral. If this is so, then one would expect saturated acyl-CoAs (produced at the end of each turn of the catalytic spiral) and unsaturated acyl-CoAs (produced by acyl-CoA dehydrogenase) to accumulate, although, if the acyl-CoA dehydrogenase is rate-limiting, one would not expect significant accumulation of enoyl-CoAs. Thus the results obtained with *Corynebacterium* sp. strain 7E1C indicate that *in vivo* the saturated acyl-CoAs arising during β -oxidation behave as free intermediates with no evidence for any form of substrate channelling, although such channelling of the other acyl-CoA intermediates cannot be discounted. It is possible that *in vivo* there would not be such an accumulation of acyl-CoAs, as the degree of accumulation would be limited by the amount of intracellular CoA. However, it is clear that the *Corynebacterium* sp. strain 7E1C β -oxidation system behaves significantly differently, with respect to acyl-CoA intermediate formation, from both mammalian mitochondrial and peroxisomal systems.

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