Comparison of metabolic fluxes of *cis*-5-enoyl-CoA and saturated acyl-CoA through the β-oxidation pathway

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The metabolic fluxes of *cis*-5-enoyl-CoAs through the β-oxidation cycle were studied in solubilized rat liver mitochondrial samples and compared with saturated acyl-CoAs of equal chain length. These studies were accomplished using either spectrophotometric assay of enzyme activities and/or the analysis of metabolites and precursors using a gas chromatographic method after conversion of CoA esters into their free acids. *Cis*-5-enoyl-CoAs were dehydrogenated by acyl-CoA oxidase or acyl-CoA dehydrogenases at significantly lower rates (10–44%) than saturated acyl-CoAs. However, enoyl-CoA hydratase hydrated *trans*-2-*cis*-5-enoyl-CoA at a faster rate (at least 1.5-fold) than *trans*-2-enoyl-CoA. The combined activities of 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase for 3-hydroxy-*cis*-5-enoyl-CoAs derived from *cis*-5-enoyl-CoAs were less than 40% of the activity for the corresponding 3-hydroxyacyl-CoAs prepared from saturated acyl-CoAs. Rat liver mitochondrial β-oxidation enzymes were capable of metabolizing *cis*-5-enoyl-CoA via one cycle of β-oxidation to *cis*-3-enoyl-CoA with two less carbons. However, the overall rates of one cycle of β-oxidation, as determined with stable-isotope-labelled tracer, was only 15–25%, for *cis*-5-enoyl-CoA, of that for saturated acyl-CoA. In the presence of NADPH, the metabolism of *cis*-5-enoyl-CoAs was switched to the reduction pathway. Therefore, in intact liver mitochondria, where the NADPH/$NADP^+$ and NAD^+/NADH ratios are high, the predominant pathway for the metabolism of *cis*-5-enoyl-CoAs could be reduction via 2,4-dienoyl-CoA reductase after isomerization of $\Delta^3\Delta^5$-dienoyl-CoA to *trans*-2-*trans*-4-dienoyl-CoA.

INTRODUCTION

Fatty acids with odd-numbered double bonds are thought to be metabolized by β-oxidation cycles to *cis*-5-enoyl-CoA, compound (I), which continues to *cis*-3-enoyl-CoA, compound (IV), via one more cycle (Figure 1). Δ^3,Δ^2-Enoyl-CoA isomerase then converts *cis*-3-enoyl-CoA into *trans*-2-enoyl-CoA and back to the regular β-oxidation cycles. This is termed the conventional pathway here. Recently, we described a new pathway for the metabolism of *cis*-5 unsaturated fatty acids [1]. This pathway requires NADPH as cofactor for the reduction of double bonds. Most recent data have indicated that this pathway is via the dehydrogenation of *cis*-5-enoyl-CoA to *trans*-2-*cis*-5-dienoyl-CoA, compound (II), which is isomerized to $\Delta^3\Delta^5$-dienoyl-CoA, compound (III-R), catalysed by Δ^3,Δ^2-enoyl-CoA isomerase or a peroxisomal trifunctional enzyme [2,3]. A new isomerase, $\Delta^3\Delta^5$-*trans*-2-*trans*-4-dienoyl-CoA isomerase, then converts $\Delta^3\Delta^5$-dienoyl-CoA into *trans*-2-*trans*-4-dienoyl-CoA, compound (IV-R), which is reduced by 2,4-dienoyl-CoA reductase with NADPH as cofactor to *trans*-3-enoyl-CoA. Another isomerization catalysed by Δ^3,Δ^2-enoyl-CoA isomerase converts *trans*-3-enoyl-CoA into *trans*-2-enoyl-CoA and back to the regular fatty acid β-oxidation cycle [2]. This is here referred to as the reduction pathway. The new enzyme, $\Delta^3\Delta^5$-*trans*-2-*trans*-4-dienoyl-CoA isomerase, has been isolated and purified to apparent homogeneity [3,4]. Although it is evident that rat liver mitochondria are capable of metabolizing *cis*-5-enoyl-CoA via the reduction pathway, the relative contribution of this pathway to the overall metabolic flux of *cis*-5-enoyl-CoA is not known. The other question to be answered is whether the conventional isomerase-mediated pathway exists in mitochondria. Although the isomerization of *cis*-3-enoyl-CoA to *trans*-2-enoyl-CoA has been demonstrated in rat liver mitochondria [5], the metabolism of *cis*-5-enoyl-CoA to *cis*-3-enoyl-CoA via one cycle of β-oxidation in the conventional pathway has never been documented. We found that the metabolism of *cis*-5-enoyl-CoA in solubilized rat liver mitochondria was extremely slow and no metabolites from a complete cycle of β-oxidation could be detected [1]. In contrast, the saturated acyl-CoAs readily produced metabolites from one to three cycles of β-oxidation under identical conditions.

In this investigation, we compare the metabolic flux through the β-oxidation cycle of *cis*-5-enoyl-CoA with that of the saturated acyl-CoA of equal chain length. We used spectrophotometric assay of enzyme activities and/or analysis of metabolites using gas chromatography after conversion of CoA esters into their free acids. The maximal catalytic activities of the β-oxidation-cycle enzymes, i.e. acyl-CoA oxidase, acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, toward *cis*-5-enoyl-CoA were measured. In addition, the overall rate of the one cycle of β-oxidation using *cis*-5-dodecenoyl-CoA as substrate in solubilized rat liver mitochondria was compared with dodecanoyl-CoA using a novel stable-isotope tracer technique. The purpose of these studies was to characterize the steps that account for the significantly lower metabolic rate of further oxidation of *cis*-5-enoyl-CoA through the β-oxidation cycle. Reconstitution experiments with purified enzymes coupled with gas-chromatographic and gas-chromatographic–mass-spectrometric analysis were used to investigate the conversion of *cis*-5-enoyl-CoA into *cis*-3-enoyl-CoA via one cycle of β-oxidation in solubilized rat liver mitochondria.

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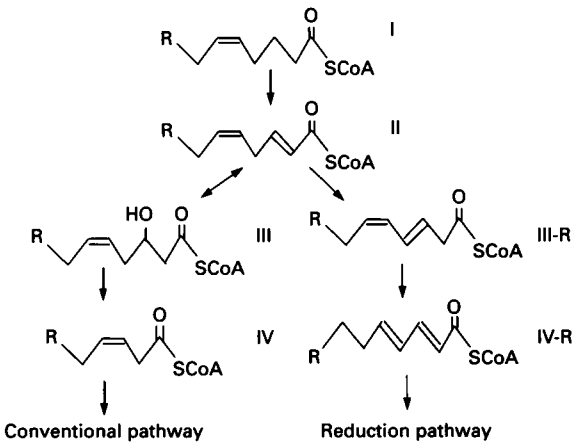

Figure 1 Metabolic pathways of unsaturated fatty acids with odd-numbered double bonds

MATERIALS AND METHODS

Reagents and chemicals

Cis-5-decenoic, *cis*-5-dodecenoic and *cis*-5-tetradecenoic acids were synthesized as previously described [6]. *Trans*-2-*trans*-4-decadienoic acid was synthesized by the oxidation of *trans*-2-*trans*-4-decadienal (Aldrich) with Jones' reagent. *Cis*-3-decenoic acid was prepared by the oxidation and reduction of *cis*-3-decyn-1-ol (Farchan) with Jones' reagent in acetone [6]. Acyl-CoA esters were prepared by the modified mixed-anhydride method with ethyl chloroformate and triethylamine in tetrahydrofuran [7]. Their concentrations were determined by Ellman's procedure [8]. Acyl-CoA oxidase (from *Arthrobacter* sp.) was purchased from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Crotonase (from porcine heart) and 3-hydroxyacyl-CoA dehydrogenase (from porcine heart) were obtained from Sigma. Dodecanoyl-CoA and decanoyl-CoA were purchased from Sigma. Tetradecanoyl-CoA was synthesized by the mixed-anhydride method [7]. NAD^+, NADPH and CoA were obtained from Sigma. [1,2,3,4-$^{13}C_4$]Dodecanoic acid was custom-synthesized by MSD Isotopes (Montreal, Que., Canada).

Partial purification of general 3-ketoacyl-CoA thiolase from rat liver homogenate

Enzymes were extracted from rat liver homogenate and fractionated with $(NH_4)_2SO_4$ (30–80 %). The dialysate was then applied to a column (1.6 cm × 30 cm) of Matrex gel Red A. After being washed with 200 ml of buffer A (20 mM phosphate buffer, pH 7.0, containing 0.5 mM benzamidine, 0.5 mM dithiothreitol, 0.5 mM EDTA and 0.5 mM EGTA), the column was eluted with a linear gradient of 0–1.2 M KCl in buffer A at a flow of 30 ml/h. The total volume of the gradient was 300 ml. Fractions containing ketothiolase were pooled and applied to a column (2.6 cm × 19 cm) of Blue Sepharose CL-6B. This column was eluted with buffer A at a flow rate of 30 ml/h. Unretained fractions contained activity for general 3-ketoacyl-CoA thiolase when assayed with 3-oxo-octanoyl-CoA as substrate.

Preparation of cholate-solubilized rat liver mitochondria

Fresh rat liver was minced and homogenized in a mixture of MSM buffer (220 mM mannitol, 70 mM sucrose, 5 mM Mops, pH 7.4) with EDTA (2 mM). The homogenate was centrifuged at 400 *g* to remove debris and then centrifuged further at 7000 *g* to obtain pellets of mitochondria. These mitochondria pellets were washed twice with MSM buffer, then suspended in the same solution. Mitochondrial enzyme extract was prepared by solubilizing mitochondria with 0.1 M phosphate buffer, pH 7.2, containing 5 % cholate to a final concentration of 1 mg of mitochondrial protein/ml and 1 % cholate.

Measurement of acyl-CoA oxidase and acyl-CoA dehydrogenase activity

Acyl-CoA oxidase (5 m-units of commercial source) activity was measured by the increase in absorbance at 260 nm using *cis*-5-enoyl-CoA or acyl-CoA (40 μM) as substrate at 37 °C; the reaction was initiated by the addition of substrate. Acyl-CoA dehydrogenases were determined spectrophotometrically at 550 nm with phenazine ethosulphate and cytochrome *c* as electron acceptor using cholate-solubilized rat liver mitochondria (40 μg of protein) as enzyme source [9]. The substrate concentration was 0.2 mM and the activity of acyl-CoA dehydrogenases in these preparations was 119 ± 16 m-units/mg when decanoyl-CoA was used as substrate.

Assay of enoyl-CoA hydratase

As the substrates, *trans*-2-*cis*-5-dienoyl-CoAs, were not available by chemical synthesis, the activity of enoyl-CoA hydratase, i.e. hydration of *trans*-2-*cis*-5-dienoyl-CoA and *trans*-2-enoyl-CoA to their 3-hydroxy metabolites, was studied using a gas-chromatographic method. The substrates were generated by dehydrogenation of *cis*-5-dodecenoyl-CoA and dodecanoyl-CoA with acyl-CoA oxidase (0.5 unit/ml). Purified crotonase (0.4 unit) was then added and the mixture was incubated at 37 °C for 5 min. The production of 3-hydroxy metabolite was determined by gas-chromatographic analysis after extraction of hydrolysed products and derivatization.

Measurement of 3-hydroxyacyl-CoA dehydrogenase activity

Measurement of the forward reaction as described by He et al. [10] was adopted. The substrates (3-hydroxyacyl-CoAs) were produced by preincubation of either *cis*-5-enoyl-CoA (40 μM) or the corresponding saturated acyl-CoA (40 μM) of the same chain length with acyl-CoA oxidase (0.5 unit) and crotonase (2 unit) at 37 °C in 20 mM phosphate buffer (pH 8) in a 1 ml cuvette for 10 min. NAD^+ (2 mM) and CoA (0.3 mM) were then added. A baseline measurement was obtained after 1 min of incubation. 3-Hydroxyacyl-CoA dehydrogenase (1 unit) or mitochondrial enzyme extract (25 μg of protein) was added to induce the reaction. The conversion of NAD^+ into NADH was measured at 340 nm. Partially purified 3-ketoacyl-CoA thiolase (10 μl containing 6.5 m-units of ketothiolase assayed with 3-oxo-octanoyl-CoA) was added together with 3-hydroxyacyl-CoA dehydrogenase in experiments using purified 3-hydroxyacyl-CoA dehydrogenase [10].

Metabolic studies

The studies were carried out in 25 ml Erlenmeyer flasks in a metabolic shaking incubator (140 cycles/min) at 37 °C. Each flask contained acyl-CoA, acyl-CoA oxidase and purified enzymes or cholate-solubilized mitochondria in either 20 mM phosphate buffer (pH 8.0) or 0.1 M phosphate buffer (pH 7.4). The reaction was stopped by the addition of 1 M KOH (120 μl/ml of incubation mixture).

Metabolic profiling of the incubation mixture

An aliquot (1 ml) of the incubation mixture, pretreated with KOH, was mixed with internal standard (20 μg of pentadecanoic acid) and hydrolysed at room temperature for 50 min. The mixture was acidified and extracted with ethyl acetate/diethyl ether (1:1). After conversion into trimethylsilyl derivatives, the sample was analysed with a dual-capillary column gas chromatograph (model 5890; Hewlett–Packard, Avondale, PA, U.S.A.), using a bonded dimethylpolysiloxane phase (SPB-1 from Supelco, Bellefonte, PA, U.S.A.) and a bonded 35% diphenyl/65% dimethylpolysiloxane phase (SPB-35)-fused silica capillary column (also available from Supelco by custom order). The initial column temperature was 60 °C and increased at 4 °C/min to 250 °C with a 50:1 split injection ratio. Quantification was based on the relative peak area to internal standard. The amount of metabolite was calculated as the weight equivalent to that of internal standard on the basis of area ratio.

A Hewlett–Packard 5985B gas chromatograph/mass spectrometer was used for metabolite identification and determination of isotope enrichment. A shorter (12.5 m) fused silica capillary column (SPB-1) was used with the same temperature program as described for gas chromatography. Electron-impact (70 eV) ionization and repetitive-scanning (300 atomic mass units/s) from m/z 49 to 550 was used to obtain mass spectra. The criteria for identification of compounds were that the retention times on both gas-chromatographic columns and the mass spectrum were identical with those obtained with authentic samples.

Selected ion-monitoring determination of isotopic enrichment in metabolites

Derivatized samples were separated through an SPB-1 capillary gas-chromatographic column as described above. The column temperature was 140 °C initially, then it was increased at a rate of 4 °C/min after injection. Chromatographic effluent was monitored at m/z 229, 231 and 233 at a dwell time of 100 ms for each ion for decanoic acids; m/z 233, 235 and 237 were used for 3-hydroxydecanoic and 3-hydroxydodecanoic acids. Peaks areas were determined and normalized to the peak area of unlabelled ion (taken as 100). Metabolites produced by the incubation of unlabelled substrate were also analysed to obtain natural abundance isotope enrichments. For the calculation of percentage enrichment of each labelled species, the relative peak areas were corrected for the contribution of natural isotope abundance by a technique described previously [11].

RESULTS

Dehydrogenation of *cis*-5-enoyl-CoA by acyl-CoA dehydrogenase and acyl-CoA oxidase

The rate of dehydrogenation of *cis*-5-dodecenoyl-CoA by rat liver mitochondria was only 44±11% ($n = 6$) of that of dodecanoyl-CoA. The rate of dehydrogenation of *cis*-5-decenoyl-CoA was only 35±2% ($n = 3$) of that with an equal concentration of decanoyl-CoA as substrate. When *cis*-5-tetradecenoyl-CoA was used as substrate, the activity was 34% of that with tetradecanoyl-CoA as substrate.

The rate of dehydrogenation of *cis*-5-decenoyl-CoA by acyl-CoA oxidase from *Arthrobacter* was only 36% of that of decanoyl-CoA. Likewise, the rate of dehydrogenation of *cis*-5-tetradecenoyl-CoA was 10% of that of tetradecanoyl-CoA. Therefore *cis*-5-enoyl-CoAs were poorer substrates for the dehydrogenation reaction, whether rat liver acyl-CoA dehydrogenases or *Arthrobacter* acyl-CoA oxidase were used. On average, the dehydrogenation rates were 35% of that with saturated acyl-CoAs when *cis*-5-enoyl-CoAs were used as substrates.

Table 1 Activities of rat liver mitochondrial 3-hydroxyacyl-CoA dehydrogenases toward 3-hydroxy-*cis*-5-enoyl-CoAs and saturated 3-hydroxyacyl-CoAs

The substrates, i.e. 3-hydroxyacyl-CoAs and 3-hydroxy-*cis*-5-enoyl-CoAs, were produced by incubation of acyl-CoA or *cis*-5-enoyl-CoA (40 μM) with acyl-CoA oxidase (0.5 unit) and crotonase (2 units) in 1 ml of 20 mM phosphate buffer, pH 8, at 37 °C. Data shown are means ± S.D. from mitochondria isolated from five rats. Abbreviation: MC, monocarboxylic acid.

	3-Hydroxyacyl-CoA dehydrogenase activity (m-units/mg of protein)		
Chain length...	MC14	MC12	MC10
Cis-5	125±31	150±36	182±44
Saturated	208±60	188±39	582±140
P (paired *t* test)	0.0036	0.0102	0.0005

Hydration of *trans*-2-*cis*-5-dienoyl-CoA by crotonase

After a 5 min incubation the amount of 3-hydroxy-*cis*-5-dodecenoate produced was 59±24% ($n = 4$) higher than the amount of 3-hydroxydodecanoate produced when equal amounts (66 μM) of *cis*-5-dodecenoyl-CoA and dodecanoyl-CoA were used as substrate with an excess of acyl-CoA oxidase. The ratio of 3-hydroxy-*cis*-5-dodecenoate to *trans*-2-*cis*-5-dodecadienoate was 5.5±3.5, whereas that of 3-hydroxydodecanoate to *trans*-2-dodecenoate was 0.6±0.2 ($P = 0.03$, $n = 4$). The equilibrium ratio (obtained after 30–60 min of incubation) for 3-hydroxy-*cis*-5-dodecenoate/*trans*-2-*cis*-5-dodecenoate was 15±2 ($n = 6$), whereas that of 3-hydroxydodecanoate/*trans*-2-dodecenoate was 4±1 ($n = 6$). After 5 min of incubation with *cis*-5-dodecenoyl-CoA as substrate the equilibrium between 3-hydroxy and *trans*-2 fatty acids was 37%; the corresponding value for dodecanoyl-CoA was 15%. Therefore the hydration of *trans*-2-*cis*-5-dodecadienoyl-CoA to 3-hydroxy-*cis*-5-dodecenoyl-CoA comprised at least 159±24% of the reactions involving *trans*-2-dodecenoyl-CoA as substrates.

3-Hydroxyacyl-CoA dehydrogenase activity toward 3-hydroxy-*cis*-5-enoyl-CoAs

Rat liver mitochondrial 3-hydroxyacyl-CoA dehydrogenase activity toward 3-hydroxy-*cis*-5-decenoyl-CoA, 3-hydroxy-*cis*-5-dodecenoyl-CoA and 3-hydroxy-*cis*-5-tetradecenoyl-CoA was measured and compared with the activity toward their saturated counterparts. The data are shown in Table 1. In all three substrates, the rates with the 3-hydroxy-*cis*-5-substrates were significantly lower than with their saturated counterparts: 61±9% for 3-hydroxy-*cis*-5-tetradecenoyl-CoA, 80±10% for 3-hydroxy-*cis*-5-dodecenoyl-CoA and 31±5% for 3-hydroxy-*cis*-5-decenoyl-CoA relative to their individual saturated counterparts. The activities of commercial porcine heart 3-hydroxyacyl-CoA dehydrogenase were 24% for 3-hydroxy-*cis*-5-tetradecenoyl-CoA, 15% for 3-hydroxy-*cis*-5-dodecenoyl-CoA and 24% for 3-hydroxy-*cis*-5-decenoyl-CoA compared with their saturated counterparts.

Conversion of 3-hydroxy-*cis*-5-enoyl-CoA into *cis*-3-enoyl-CoA by one cycle of β-oxidation

The combined activity of 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase was studied by measuring the

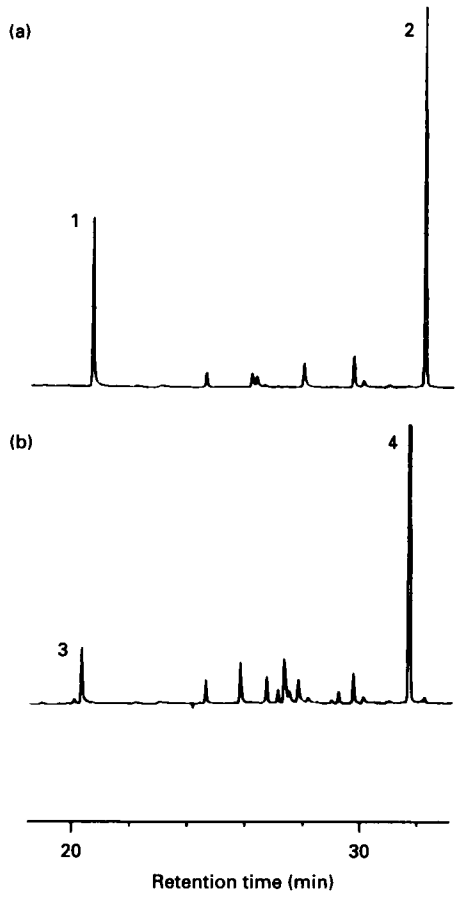

Figure 2 (a) Conversion of 3-hydroxydodecanoyl-CoA (74 μM) into decanoate after β-oxidation catalysed by 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase at 37 °C, and (b) the metabolic conversion of 3-hydroxy-*cis*-5-dodecenoyl-CoA (78 μM) into *cis*-3-decenoate under similar conditions

Metabolites: 1, decanoate; 2, 3-hydroxydodecanoate; 3, *cis*-3-decenoate; 4, 3-hydroxy-*cis*-5-dodecenoate. The metabolites produced were 27 μM decanoate and 11 μM *cis*-3-decenoate in this experiment. 3-Hydroxy-*cis*-5-dodecenoyl-CoA was produced by incubation of *cis*-5-dodecenoyl-CoA with acyl-CoA oxidase (0.5 unit) and crotonase (0.56 unit) in 20 mM phosphate buffer, pH 8, for 10 min. Partially purified rat liver 3-ketoacyl-CoA thiolase (33 m-units using 3-oxo-octanoyl-CoA as substrate), porcine heart 3-hydroxyacyl-CoA dehydrogenase (1.2 units), CoA (0.3 mM) and NAD^+ (2 mM) were then added and incubation was continued for an additional 2 min. The mixtures were hydrolysed and analysed by dual-column capillary gas chromatography. The conditions for the 3-hydroxydodecanoyl-CoA experiment were similar to the above conditions except that acyl-CoA oxidase was not added.

production of *cis*-3-enoyl-CoA from 3-hydroxy-*cis*-5-enoyl-CoA. In these experiments, bovine liver crotonase was used to generate 3-hydroxy-*cis*-5-dodecenoyl-CoA from *trans*-2-*cis*-5-dodecadienoyl-CoA produced from *cis*-5-dodecenoyl-CoA and acyl-CoA oxidase. To this mixture was then added 3-hydroxyacyl-CoA dehydrogenase and partially purified rat liver general 3-ketoacyl-CoA thiolase. The results are shown in Figure 2. *Cis*-3-decenoate was formed. Therefore enzymes from rat liver mitochondria are capable of metabolizing *cis*-5-enoyl-CoA to *cis*-3-enoyl-CoA by the conventional isomerase pathway via one cycle of β-oxidation. The quantitative aspect of this conversion was compared with similar studies performed on 3-hydroxydodecanoyl-CoA. Decanoate was produced faster than the corresponding reaction to *cis*-3-decenoate with a product ratio, after 2 min incubation, of

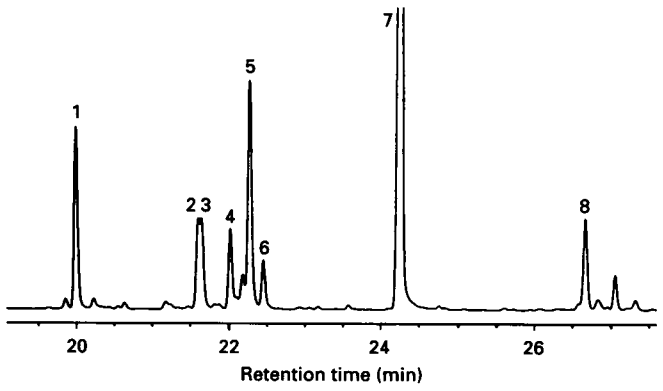

Figure 3 Metabolites produced by incubation of *cis*-5-decenoyl-CoA (66 μM) with acyl-CoA oxidase (0.5 unit) and rat liver mitochondria (1 mg of protein) in 1 ml of 0.1 M phosphate buffer, pH 7.4, at 37 °C for 15 min

Metabolites: 1, *cis*-5-decenoate; 2, *cis*-3-*trans*-5-decadienoate; 3, *trans*-3-*cis*-5-decadienoate; 4, *cis*-3-*cis*-5-decadienoate; 5, *trans*-3-*trans*-5-decadienoate; 6, *trans*-2-*cis*-4-decadienoate; 7, *trans*-2-*trans*-4-decadienoate; 8, 3-hydroxy-*cis*-5-decadienoate. This metabolite profile was analysed on a non-polar SPB-1 capillary column. The identities of the metabolites were also confirmed by retention on another polar capillary column (SPB-35); in this column, peaks 2 and 3 were completely separated.

Table 2 Major metabolites (in μM) from the incubation of *cis*-5-decenoyl-CoA (66 μM) with rat liver mitochondrial enzyme extract (1 mg of protein/ml) at 37 °C

Abbreviations: c5MC10, *cis*-5-decenoate; 3OHc5MC10, 3-hydroxy-*cis*-5-decenoate; t2t4MC10, *trans*-2-*trans*-4-decadienoate; t3c5MC10, *trans*-3-*cis*-5-decadienoate; t3t5MC10, *trans*-3-trans-5-decadienoate. Data shown are means ± S.D. from mitochondria isolated from five rats. Metabolites were analysed by gas chromatography after alkaline hydrolysis.

Time (min)	c5MC10	3OHc5MC10	t2t4MC10	t3c5MC10	t3t5MC10
1	55 ± 5	9.0 ± 0.5	0.9 ± 0.3	1.2 ± 0.3	0 ± 0
2	38 ± 1	13.3 ± 2.1	3.2 ± 0.6	3.4 ± 0.5	1.0 ± 0.3
5	25 ± 3	9.9 ± 3.3	7.7 ± 1.0	5.1 ± 1.5	2.2 ± 0.3
10	20 ± 3	6.1 ± 1.9	12.2 ± 3.1	4.1 ± 1.0	3.4 ± 0.7

2.5 to 1. Therefore the combined rate of dehydrogenation and thiolase cleavage was 40%, for 3-hydroxy-*cis*-5-dodecenoyl-CoA, of that with 3-hydroxydodecanoyl-CoA as substrate. Under similar conditions, incubation of 3-hydroxy-*cis*-5-tetradecenoyl-CoA yielded *cis*-3-dodecenoate at a rate that was only 32% of the production of dodecanoate from an equal amount of 3-hydroxytetradecanoyl-CoA. The production of *cis*-3-dodecenoate was linear from 2 to 10 min of incubation.

Metabolism of *cis*-5-enoyl-CoA in solubilized rat liver mitochondria

When *cis*-5-decenoyl-CoA was incubated with acyl-CoA oxidase and solubilized rat liver mitochondria, the major metabolite formed was *trans*-2-*trans*-4-decadienoate as shown in Figure 3. The time course of the production of metabolites is shown in Table 2. After short periods of incubation, 3-hydroxy-*cis*-5-decenoate was the major metabolite. However, after prolonged incubation, the major metabolite was *trans*-2-*trans*-4-decadi-

enoate at the expense of 3-hydroxy-*cis*-5-decenoate. *Trans*-3-*cis*-5-decadienoate and *trans*-3-*trans*-5-decadienoate were the predominant $\Delta^3\Delta^5$-dienoates accumulated with time. The metabolites that accumulated in the incubation mixture could not account completely for the disappearance of *cis*-5-decenoyl-CoA even though no metabolites from one complete cycle of β-oxidation could be found.

In comparison, decanoyl-CoA was metabolized to 3-hydroxydecanoate and 3-hydroxyoctanoate. The latter metabolite was apparently derived from 3-hydroxydecanoyl-CoA after one complete β-oxidation cycle.

Determination of metabolic pathway by using stable-isotope-labelled tracer

Cis-5-dodecenoyl-CoA and [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA were co-incubated with solubilized rat liver mitochondria (by freezing and thawing) to determine the flux of *cis*-5-dodecenoyl-CoA through the conventional β-oxidation cycle. The rationale of this study design was that if *cis*-5-dodecenoyl-CoA completed one cycle of β-oxidation to *cis*-3-decenoyl-CoA, this metabolic intermediate would then immediately be isomerized to *trans*-2-decenoyl-CoA, which would be hydrated to 3-hydroxydecanoyl-CoA. 3-Hydroxydecanoyl-CoA formed by the oxidation of dodecanoyl-CoA would function as a carrier of 3-hydroxydecanoyl-CoA produced by the oxidation of *cis*-5-dodecenoyl-CoA and thereby allow detection. The two 3-hydroxydecanoyl-CoA sources can be distinguished by their ^{13}C labelling: 3-hydroxydecanoate formed from *cis*-5-dodecenoyl-CoA would have no ^{13}C enrichment, whereas that from dodecanoyl-CoA would contain [1,2-$^{13}C_2$] labelling. Selected ion-monitoring determination of $M+0$ and $M+2$ species would distinguish between the contributions from each pathway.

Incubation of [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA (77 μM) alone with acyl-CoA oxidase and solubilized rat liver mitochondria yielded 3-hydroxydodecanoate (21 μM), 3-hydroxydecanoate (17 μM), 3-hydroxyoctanoate (17 μM), a minor amount of decanoate, *trans*-2-decanoate, *trans*-2-dodecanoate and unchanged dodecanoate (total 22 μM). In this study, the $M+4$ enrichment of 3-hydroxydodecenoate was 100%, the $M+2$ enrichment of 3-hydroxydecanoate was 98%, and $M+2$ enrichment of decanoate was 97%. From these isotope-enrichment experiments, the product–precursor relationship between dodecanoyl-CoA and 3-hydroxydodecanoate, 3-hydroxydecanoate and decanoate was established. The small dilution of isotope enrichments probably resulted from the entry of unlabelled decanoate from tissue fatty acids.

The co-incubation of equal amounts of [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA and *cis*-5-dodecanoyl-CoA (77 μM each) yielded metabolites with a slight dilution of 3-hydroxydodecanoate (99% $M+4$). The $M+0$ enrichment of 3-hydroxydecanoate ranged from 3 to 15% (11 ± 5%) for incubation times of 2–10 min. In these experiments, the conversion of 3-hydroxy-*cis*-5-dodecenoyl-CoA into *cis*-3-decenoyl-CoA, then to 3-hydroxydecanoyl-CoA, was not dependent on the substrate concentration of precursor. On the basis of these data, the β-oxidation rate of the first complete cycle for *cis*-5-dodecenoyl-CoA was only 11 ± 5% of that for dodecanoyl-CoA. Decanoate produced in these experiments had an $M+0$ enrichment of 4 ± 2%, not significantly different from that obtained with labelled dodecanoyl-CoA alone. This indicated that the reduction pathway was not operating under the uncoupled conditions in freeze–thawed mitochondria. Unlabelled decanoate can only be produced by the reduction pathway. The addition of NAD^+ (1.5 mM) increased the $M+0$ enrichment of 3-hydroxydecanoate to 24 ± 5% ($P = 0.001$; t test). Therefore, a high NAD^+/NADH ratio increased the conventional pathway, but the overall flux was still significantly below that for saturated acyl-CoA.

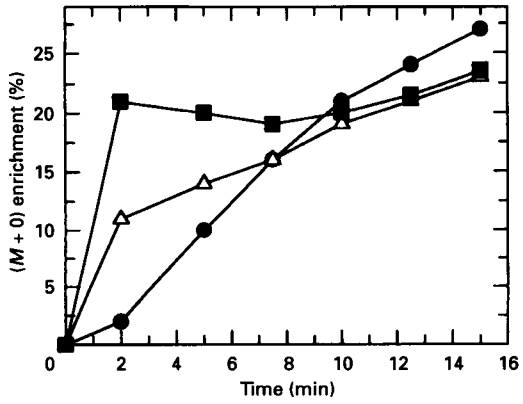

Figure 4 Isotope enrichments (M + 0, %) of 3-hydroxydodecanoate (●), 3-hydroxydecanoate (△) and decanoate (■) produced by the incubation of equal amounts of [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA and *cis*-5-dodecenoyl-CoA (77 μM each) with acyl-CoA oxidase (0.5 unit) and rat liver mitochondria (1 mg of protein) in 1 ml of 0.1 M phosphate buffer, pH 7.4, in the presence of NADPH (0.7 mM)

Unlabelled metabolites ($M+0$) were derived from the metabolism of *cis*-5-dodecenoyl-CoA, and those derived from dodecanoyl-CoA had either $M+4$ or $M+2$ labels.

When NADPH (0.7 mM) was included in the incubation mixture, the $M+0$ enrichment in 3-hydroxydodecanoate increased with incubation time as shown in Figure 4. The concentration of 3-hydroxy-*cis*-5-dodecenoate decreased from 40 μM at 2 min to 9 μM at 15 min of incubation and the concentration of 3-hydroxydodecanoate stayed constant at 71 ± 3 μM. These data indicate that the reduction pathway converted 3-hydroxy-*cis*-5-dodecenoyl-CoA into 3-hydroxydodecanoyl-CoA. In contrast with the incubation without NADPH (Table 2), the concentration of *trans*-2-*trans*-4-dodecadienoate did not change significantly, staying at 2 ± 1 μM. The incubation with both NADPH (0.7 mM) and NAD^+ (1.5 mM) gave the same results as the experiments with NADPH alone.

DISCUSSION

In this study, we have shown that *cis*-5-enoyl-CoAs were dehydrogenated at lower rates than the corresponding saturated acyl-CoAs. The hydration of *trans*-2-*cis*-5-enoyl-CoA to 3-hydroxy-*cis*-5-enoyl-CoA mediated by crotonase was faster than the same reaction using *trans*-2-enoyl-CoA as substrate. The dehydrogenation of 3-hydroxy-*cis*-5-enoyl-CoA and the cleavage to form *cis*-3-enoyl-CoA with two less carbons was also shown to be slower (33%) than with the corresponding saturated acyl-CoA. The decrease in the rate of this conversion could be due, at least in part, to the 3-hydroxyacyl-CoA dehydrogenase, as enzyme assay revealed a reduced activity of this enzyme with 3-hydroxy-*cis*-5-enoyl-CoAs as substrates. However, even with these reduced enzyme activities toward *cis*-5-enoyl-CoA and intermediates, the mitochondrial enzymes were capable of metabolizing *cis*-5-enoyl-CoA to *cis*-3-enoyl-CoA via one cycle of β-oxidation in the absence of the competing reduction pathway. However, the rate of this overall conversion was much slower than that of the corresponding saturated acyl-CoA. The failure to detect metabolites in previous studies was due to the rate of

production of these metabolites being too low to permit their accumulation.

The branching point for the two pathways, i.e. reduction and conventional, is *trans*-2-*cis*-5-dienoyl-CoA (Figure 1). The *reversible* hydration of this intermediate by enoyl-CoA hydratase to 3-hydroxy-*cis*-5-enoyl-CoA would potentially channel the oxidation towards the conventional pathway. On the other hand, the *irreversible* isomerization of *trans*-2-*cis*-5-dienoyl-CoA to $\Delta^3\Delta^5$-dienoyl-CoA [4] would favour the reduction pathway. However, enoyl-CoA hydratase is not a rate-limiting enzyme in fatty acid β-oxidation. The metabolic fluxes appear to be determined by the step(s) after the formation of 3-hydroxyacyl-CoA, i.e. 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase [12]. For *cis*-5-enoyl-CoA, these two steps have been shown in this investigation to operate at no more than one-third of the activity observed for saturated acyl-CoA. The overall pathway, as measured by stable-isotope labelling, indicated that *cis*-5-enoyl-CoAs were metabolized by one cycle of β-oxidation at a rate that was less than 15–25% of that for saturated acyl-CoA.

In the presence of the NADPH, the metabolism of *cis*-5-enoyl-CoA switched to the reduction pathway. *Trans*-2-*trans*-4-dodecadienoate as CoA ester, which normally accumulated in the absence of NADPH (Table 2), did not accumulate and was converted into 3-hydroxydodecanoyl-CoA, through *trans*-3-enoyl-CoA and *trans*-2-enoyl-CoA. 3-Hydroxydodecanoyl-CoA was then metabolized via the β-oxidation pathway to decanoyl-CoA and 3-hydroxydecanoyl-CoA. As shown in Figure 4, the $M+0$ enrichments of decanoate stayed at 20%. This indicated that 20% of decanoate was derived from *cis*-5-dodecenoyl-CoA by the reduction pathway, as the conventional pathway does not produce decanoate. The rest of the decanoate (80%) with $M+2$ label was derived from the β-oxidation of $M+4$ labelled dodecanoyl-CoA. Likewise, unlabelled 3-hydroxydodecanoate was produced from *cis*-5-dodecenoyl-CoA by the reduction pathway. However, unlabelled ($M+0$) 3-hydroxydecanoate can be produced by either the conventional or the reduction pathway. After 8 min of incubation, the $M+0$ content of 3-hydroxydodecanoate, 3-hydroxydecanoate and decanoate became equal and showed product–precursor relationship. This is consistent with the reduction pathway being the major route of metabolism in the presence of NADPH. In these studies, the smaller contribution of unlabelled decanoate and 3-hydroxydodecanoate to the overall pools of these metabolites produced by the reduction of an equal concentration of *cis*-5-dodecenoyl-CoA can be rationalized by the lower production rates. The sequence for dodecanoyl-CoA leading to 3-hydroxydodecanoate required only two steps, i.e. dehydrogenation and hydration. In comparison, the sequence for *cis*-5-dodecenoyl-CoA required six to eight steps, i.e. dehydrogenation, isomerization, isomerization, reduction, isomerization and hydration, or dehydrogenation, hydration, dehydration, isomerization, isomerization, reduction, isomerization and hydration. At early incubation times (Figure 4), the $M+0$ enrichments of decanoate and 3-hydroxydecanoate were significantly higher than that of 3-hydroxydodecanoate. This is probably caused by the much larger 3-hydroxydodecanoate pool; therefore it took longer for this pool to be equilibrated. Such a relationship is consistent with the 'leaking hosepipe' model proposed by Stanley and Tubbs [13].

In intact mitochondria, the rates of oxidation of fatty acids containing even- and odd-numbered double bonds have been shown to be comparable with those of saturated fatty acids [14]. In the present study, the β-oxidation rate of *cis*-5-enoyl-CoA in solubilized mitochondria, which lacked the reduction pathway because of a low NADPH/$NADP^+$ ratio [15], was shown to be significantly lower than that of the corresponding saturated fatty acids. Therefore it is likely that the reduction pathway for unsaturated fatty acids with odd-numbered double bonds is important in intact mitochondria to compensate for the decreased conventional β-oxidation pathway. Rat liver mitochondria have an NAD^+/NADH ratio of 20 and an NADPH/$NADP^+$ ratio of 200 [15]. The high intramitochondrial NADPH/$NADP^+$ ratio is maintained by isocitrate dehydrogenase, glutamate dehydrogenase and energy-dependent nicotinamide nucleotide transhydrogenase [15]. It is possible that the reduction pathway of unsaturated fatty acids accounts for the requirement for high transhydrogenase and isocitrate dehydrogenase activity in heart cells despite a lack of major NADPH-requiring synthetic processes in these sites. Heart cells use fatty acids as their major metabolic fuel.

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