Estimation of peroxisomal β -oxidation in rat heart by a direct assay of acyl-CoA oxidase

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The contribution of peroxisomes to palmitate β -oxidation in rat heart was estimated by either inhibiting mitochondrial β -oxidation or measuring the activity of acyl-CoA oxidase. When respiratory inhibitors such as KCN or antimycin plus rotenone, or inhibitors of mitochondrial fatty acid uptake such as 2-tetradecylglycidic acid or 2-bromopalmitate, were used, degrees of inhibitions ranging from 24% to 87% were observed for palmitate β -oxidation by a rat heart homogenate. Although the oxidation of palmitoyl-L-carnitine by coupled rat heart mitochondria was almost completely (94%) inhibited by KCN, the inhibition by antimycin plus rotenone was incomplete (77%) and was stimulated by L-carnitine. A direct assay of acyl-CoA oxidase, based on the spectrophotometric measurement at

300 nm of 2,4-decadienoyl-CoA formation from 4-*trans*-decenoyl-CoA, was evaluated with the aim of obtaining reliable values for the activity of this enzyme, which is presumed to catalyse the rate-limiting step of peroxisomal β -oxidation. Activities determined by use of this assay were much higher than activities obtained by a coupled assay [Small, Burdett and Connock (1985) *Biochem. J.* 227, 205–210] commonly used to measure the activity of acyl-CoA oxidase. However, both methods yielded the same relative activities with different tissue homogenates. Based on an estimated palmitoyl-CoA oxidase activity of 0.3 nmol/min per mg of protein, the contribution of peroxisomes to palmitate β -oxidation in a rat heart homogenate would optimally be 4%, and most likely is several-fold lower.

INTRODUCTION

When in 1976 Lazarow and de Duve reported that rat liver peroxisomes can degrade fatty acids by β -oxidation [1], they also provided the first evidence for the coexistence of two different β -oxidation systems in animal cells. Although the intermediates formed during fatty acid β -oxidation in mitochondria and peroxisomes are identical, the enzymes of the two systems are different [2]. Moreover, the first reaction of the β -oxidation spiral in peroxisomes is catalysed by an acyl-CoA oxidase which, in contrast with the mitochondrial acyl-CoA dehydrogenases, directly reduces oxygen to H_2O_2 [3]. Because acyl-CoA oxidase from animals is virtually inactive toward short-chain substrates [3], fatty acids can be chain-shortened in peroxisomes of animals, but cannot be completely degraded. This situation has prompted many studies aimed at elucidating the metabolic function of the peroxisomal β -oxidation system in animal cells. Attempts to estimate the contribution of peroxisomal β -oxidation to the total oxidation of fatty acids in rat hepatocytes have yielded values between 5 and 30% for palmitate [4–6]. However, it has become increasingly clear that the main function of the peroxisomal β -oxidation system is the chain-shortening of fatty acids, especially of very-long-chain fatty acids such as lignoceric acid, and of related compounds such as dicarboxylic acids, prostaglandins and hydroxylated 5 β -cholestanoic acids (see [7] for a detailed review).

Heart muscle, which has a high capacity for oxidizing fatty acids, also contains peroxisomes [8] with an active β -oxidation system [9]. Estimates of the activity of peroxisomal β -oxidation in heart differ widely, depending on the method used for arriving at the estimate. Van Veldhoven and Mannaerts [10], who compared the activity of peroxisomal acyl-CoA oxidase with the rate of mitochondrial β -oxidation, concluded that peroxisomal β -oxidation of palmitate in heart is of little or no quantitative significance. In contrast, Veerkamp and van Moerkerk [11], who

compared total rates and antimycin-insensitive rates of palmitate oxidation in rat heart homogenates, estimated the peroxisomal contribution to be between 20% and 30%.

This discrepancy between published estimates of the peroxisomal contribution to fatty acid oxidation in heart prompted this study. Here we show that measurements of fatty acid oxidation with heart homogenates in the presence of inhibitors of mitochondrial β -oxidation do not provide acceptable values for the rate of peroxisomal β -oxidation. An acyl-CoA oxidase assay, based on the measurement of enoyl-CoA formation, was evaluated and shown to yield acceptable values for the activity of this enzyme. A comparison of the acyl-CoA oxidase activity with the rate of fatty acid oxidation in a rat heart homogenate leads to the conclusion that peroxisomal β -oxidation accounts at most for 4% of palmitate oxidation in heart.

MATERIALS AND METHODS

Materials

Antimycin, rotenone, CoA, L-carnitine, palmitoyl-L-carnitine, palmitoyl-CoA, decanoyl-CoA, defatted BSA, FAD, bicinchoninic acid reagent, Nycodenz, Nagarse, horseradish peroxidase, acyl-CoA oxidase from *Candida* species and all standard biochemicals were purchased from Sigma. 4-*trans*-Decenal, 4-*cis*-decenal and 2,4-*trans*-decadienal were bought from Aldrich. Fluka was the source of 3-amino-1,2,3-triazole and 2-bromopalmitic acid. Scinti Verse II was supplied by Fisher Scientific. 2,7-Dichlorofluorescein diacetate was obtained from Eastman Kodak. [1- 14 C]Palmitic acid (57.1 mCi/mmol) and [1- 14 C]palmitoylcarnitine (55 mCi/mmol) were obtained from DuPont–New England Nuclear. The following enzymes were isolated and purified by the indicated procedures: 3-ketoacyl-CoA thiolase (EC 2.3.1.16) from pig heart [12], acyl-CoA oxidase [13] and trifunctional enzyme [14], from rat liver. 2-Tetra-

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decylglycidic acid was provided by Dr. P. and Dr. L. Brady, University of Minnesota. Sprague-Dawley rats (male, 250–300 g) were obtained from Taconic Farms, Germantown, NY, U.S.A.

Preparation of substrates

4-*trans*-Decenal, 4-*cis*-decenal and 2,4-*trans*-decadienal were oxidized with Ag_2O to their respective acids as described in principle by Thomason and Kubler [15]. CoA thioesters of 4-*trans*-decenoic acid, 4-*cis*-decenoic acid, 2,4-*trans*-decadienoic acid and 2-bromopalmitic acid were synthesized by the mixed-anhydride procedure as detailed by Fong and Schulz [16]. The CoA thioesters, except for 2-bromopalmitoyl-CoA, were further purified by h.p.l.c. on a Waters μ Bondapak C_{18} reverse-phase column (30 mm $\times$ 3.9 mm) attached to a Waters gradient h.p.l.c. system. Separation was achieved by linearly increasing the acetonitrile/water (9:1, v/v) content of the 25 mM ammonium phosphate (pH 5.5) elution buffer from 20 to 50% in 20 min at a flow rate of 2 ml/min. Fractions containing the desired material were collected and, after removal of acetonitrile under reduced pressure, were passed through a Waters C_{18} Sep-pak column. The bound acyl-CoA was eluted with methanol/water (10:1, v/v) and concentrated by removing methanol under a stream of nitrogen. Concentrations of acyl-CoAs were determined by measuring released CoA as described by Ellman [17] after quantitatively cleaving the thioester bond with 1 M hydroxylamine at pH 7.

Preparation of mitochondria and rat tissue homogenates

Rat heart mitochondria were isolated by the procedure of Chappell and Hansford [18]. Rat heart homogenates for measuring fatty acid oxidation were prepared by finely cutting rat hearts with a pair of scissors and homogenizing the tissue with 50 vol. of 10 mM Tris/HCl (pH 7.4) containing 0.25 M sucrose and 2 mM EDTA, in a hand-held loose-fitting glass-Teflon homogenizer. The conditions used to prepare the rat heart homogenate were otherwise identical with those used for the isolation of mitochondria, except that the treatment with Nagarse was omitted.

Rat tissue homogenates for measuring acyl-CoA oxidase activity were prepared by placing tissues into ice-cold saline solution to wash out blood. All subsequent procedures were carried out at 0–4 °C. Minced tissues were suspended in 6 vol. of 10 mM Tris/HCl (pH 8) containing 10% glycerol, 1 mM EDTA, 0.5 mM phenylmethanesulphonyl fluoride, 0.5 mM benzamidine, aprotinin (0.06 kallikrein-inhibitor unit/ml), pepstatin A (2 $\mu\text{g}/\text{mg}$) and 0.1 mM FAD, and homogenized with a Polytron homogenizer for 3 $\times$ 20 s at close to top speed. When homogenates for assaying acyl-CoA oxidase by the method of Small et al. [19] were prepared, the same procedure was used, except that FAD was omitted from the isolation buffer. The resulting homogenates were sonicated for 3 $\times$ 20 s with an Ultrasonic sonifier (model W-385) equipped with a 1.27 cm (0.5 in) tip. Extracts of skeletal muscle, heart and brain were prepared by adding Triton X-100 to the homogenates to a final concentration of 1.5% (w/v). After keeping the suspensions for 5 min at 0 °C, they were centrifuged for 3 min at 16000 *g*, and the supernatants, referred to as extracts, were assayed for acyl-CoA oxidase.

Measurements of fatty acid oxidation with rat heart homogenate or rat heart mitochondria

For measuring the oxidation of [1- ^{14}C]palmitic acid by a rat heart homogenate, the reaction mixture contained, in a final

volume of 0.5 ml, 0.12 M KCl, 75 mM Tris/HCl (pH 7.4), 10 mM potassium phosphate, 5 mM MgCl_2 , 1 mM EDTA, 1 mM NAD^+ , 5 mM ATP, 5 mM ADP, 10 μM FAD, 100 μM CoA, 0.5 mM L-malate, 0.5 mM L-carnitine, 120 μM [1- ^{14}C]palmitic acid (100000 d.p.m.) bound to fatty-acid-free BSA and rat heart homogenate (0.5 mg wet wt. of tissue). Fatty acid oxidation was initiated by the addition of rat heart homogenate, and the rate of [1- ^{14}C]palmitic acid degradation was measured at 37 °C under shaking in a 20 ml Erlenmeyer flask covered by a rubber septum to which a plastic centre well was attached. When inhibitors were used, the homogenate was preincubated for 10 min with the inhibitory compound before it was added to the reaction mixture. After incubation for 30 min, 0.3 ml of 1 M Hyamine hydroxide was injected into the centre well, and the reaction was stopped by injection of 0.1 ml of 70% HClO_4 into the incubation medium. The flask was shaken for an additional 2 h to allow for the complete absorption of $^{14}\text{CO}_2$ by Hyamine hydroxide. The centre well was then transferred to a 7 ml scintillation vial, and the Hyamine hydroxide was thoroughly mixed with 4 ml of Scinti Verse II, before counting the resulting solution for radioactivity in a liquid-scintillation counter. For measuring ^{14}C -labelled acid-soluble products, the acidified reaction mixture was extracted three times with water-saturated butan-1-ol. A portion of the aqueous phase (0.2 ml) was mixed with 4 ml of Scinti Verse II, and the radioactivity of acid-soluble ^{14}C -labelled products were determined in a liquid-scintillation counter. Net radioactivity values were calculated by subtracting values obtained from control experiments in which reactions were immediately terminated by addition of 0.1 ml of 70% HClO_4 .

For measuring the oxidation of [1- ^{14}C]palmitoylcarnitine by rat heart mitochondria the reaction mixture contained, in a final volume of 0.7 ml, 0.11 M KCl, 33 mM Tris/HCl (pH 7.4), 2 mM potassium phosphate, 2 mM MgCl_2 , 0.1 mM EGTA, fatty-acid-free BSA (0.1 mg/ml), 0.5 mM L-malate, 3 mM ADP, 20 μM [1- ^{14}C]palmitoyl-L-carnitine (50000 d.p.m.) and rat heart mitochondria (120 μg of protein). Fatty acid oxidation was initiated by the addition of rat heart mitochondria, and the rate of [1- ^{14}C]palmitoylcarnitine degradation was measured at 25 °C under shaking in a 20 ml Erlenmeyer flask. When respiratory inhibitors were used, mitochondria were preincubated with them for 10 min before starting the reaction by addition of mitochondria to the incubation mixture. At the end of the incubation period of 6–10 min, 0.1 ml of 70% HClO_4 was added and the acidified reaction mixture was extracted three times with water-saturated butan-1-ol. A portion of the aqueous phase (0.2 ml) was mixed with 4 ml of Scinti Verse II, and the radioactivity of acid-soluble ^{14}C -labelled products was determined in a liquid-scintillation counter. Net radioactivity values were calculated by subtracting values obtained from control experiments in which reactions were immediately stopped by addition of 0.1 ml of 70% HClO_4 .

Complexes of palmitic acid, 2-bromopalmitic acid or 2-tetradecylglycidic acid with BSA were prepared by dissolving the acid in toluene, removing the solvent under a stream of nitrogen, and dissolving the resultant thin film of fatty acid in 10 mM Tris/HCl (pH 7.4) containing 1 mM EDTA and BSA under shaking for 4 h at 37 °C. The molar ratio of fatty acid to BSA was 2:1.

Oxygen uptake by rat heart mitochondria was measured at 25 °C by using a Clark oxygen electrode attached to a Gilson oxygraph. A standard incubation mixture contained, in 1.9 ml, 0.11 M KCl, 33 mM Tris/HCl (pH 7.4), 2 mM potassium phosphate, 2 mM MgCl_2 , 0.1 mM EGTA, 14.7 μM BSA, 0.5 mM L-malate, 13 mM L-carnitine, and mitochondria (1 mg of protein). After preincubation of the mixture with or without inhibitor for

1 min, the reaction was started by addition of 1 mM ADP and 15 μ M palmitoyl-L-carnitine.

Protein and enzyme assays

Protein concentrations were determined with bicinchoninic acid as described by Smith et al. [20], or by the method of Lowry et al. [21] with BSA as a standard.

Acyl-CoA oxidase was measured by three different spectrophotometric methods. (1) The substrate-dependent formation of H_2O_2 was measured in a coupled assay as described by Small et al. [19]. Activities are expressed as nmol of 2,7-dichlorofluorescein oxidized/min. (2) The formation of 2,4-decadienoyl-CoA from 4-decenoyl-CoA was measured at 300 nm as described in principle by Hovik and Osmundsen [22]. A standard assay mixture contained, in a total volume of 0.66 ml, 20 mM potassium phosphate (pH 7.4), 0.1 mM FAD, BSA (0.2 mg/ml) and acyl-CoA oxidase. When acyl-CoA oxidase was assayed in a tissue homogenate, 0.05% Triton was included and BSA was omitted. After preincubating the assay mixture for 2 min, the reaction was started by adding 50 μ M 4-*trans*-decenoyl-CoA or 4-*cis*-decenoyl-CoA. The amount of acyl-CoA oxidase was adjusted to obtain an absorbance change of approx. 0.04/min. For calculating rates, molar absorption coefficients of 27800 $M^{-1}\cdot cm^{-1}$ and 19900 $M^{-1}\cdot cm^{-1}$ were used when 2-*trans*-4-*trans*-decadienoyl-CoA [23] and 2-*trans*-4-*cis*-decadienoyl-CoA [24], respectively, were the products. When purified acyl-CoA oxidase from rat liver was assayed, the stock solution was diluted with 20 mM potassium phosphate (pH 8) containing 0.1 mM FAD and BSA (1 mg/ml). Diluted enzyme solutions were only stable for short periods of time. (3) The formation of NADH was measured at 340 nm by an assay in which enoyl-CoA formed by acyl-CoA oxidase was further β -oxidized by enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase present in a rat liver homogenate, or by the combined actions of purified peroxisomal trifunctional enzyme and 3-ketoacyl-CoA thiolase. The assay mixture contained 20 mM potassium phosphate (pH 8), 0.1 mM FAD, 1 mM NAD^+ , 0.3 mM CoA, BSA (0.2 mg/ml), 50 μ M decanoyl-CoA, and either rat liver homogenate plus 0.05% Triton X-100 or acyl-CoA oxidase plus trifunctional enzyme (0.1 mg) and 3-ketoacyl-CoA thiolase (0.3 unit). All enzyme assays were performed at 25 °C on a Gilford model 2600 recording spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that catalyses the conversion of 1 μ mol of substrate into product/min.

RESULTS

Estimation of peroxisomal fatty acid oxidation by inhibiting mitochondrial β -oxidation

Palmitate β -oxidation by a rat heart homogenate was determined as a function of time (see Figure 1a). The rate of this process, as reflected by the formation of acid-soluble products, was linear for more than 30 min when both ADP and ATP were present to support respiration and fatty acid activation, respectively. The linearity was shorter when ADP was omitted. Since the rate of CO_2 evolution was low compared with the formation of acid-soluble products, rates of β -oxidation were based only on the measured amounts of acid-soluble products. Rates of palmitate β -oxidation by a rat heart homogenate in the absence of inhibitors were close to 600 nmol of acid-soluble products formed/min per g wet wt. of tissue (see Table 1) or 7.7 nmol/min per mg of protein. This value agrees with values of 500–800 nmol/min per

Figure 1 β -Oxidation of [1- ^{14}C]palmitate by a rat heart homogenate (a) and β -oxidation of [1- ^{14}C]palmitoyl-L-carnitine by rat heart mitochondria (b)

Symbols: squares, acid-soluble products; circles, CO_2 . (a) Continuous line, products formed in the presence of ATP plus ADP; broken line, products formed when ATP was present but ADP was omitted. For experimental details see the Materials and methods section.

Table 1 Effect of inhibitors on the oxidation of [1- ^{14}C]palmitic acid by rat heart homogenate

Values are means $\pm$ S.D. based on at least three assays. For experimental details see the Materials and methods section.

Inhibitor	Acid-soluble products (nmol/min per g wet wt. of tissue)	Inhibition (%)
None	614 $\pm$ 66	0
200 μ M Antimycin + 30 μ M rotenone	285 $\pm$ 13	54
2 mM KCN	422 $\pm$ 2	31
30 μ M 2-Tetradecylglycidic acid	302 $\pm$ 16	51
120 μ M 2-Tetradecylglycidic acid	234 $\pm$ 8	62
30 μ M 2-Bromopalmitic acid	182 $\pm$ 9	70
120 μ M 2-Bromopalmitic acid	82 $\pm$ 6	87
10 μ M 2-Bromopalmitoyl-CoA	465 $\pm$ 19	24
120 μ M 2-Bromopalmitoyl-CoA	220 $\pm$ 12	64

Table 2 Effect of respiratory inhibitors and L-carnitine on the oxidation of [1- ^{14}C]palmitoylcarnitine by rat heart mitochondria

Values are means $\pm$ S.D. For experimental details see the Materials and methods section.

Inhibitor	Addition of L-carnitine (mM)	Acid-soluble products (nmol/min per mg of protein)	Inhibition (%)
None	0	1.4 $\pm$ 0.08	0
	0.5	1.46 $\pm$ 0.02	0
	2.0	1.44 $\pm$ 0.02	0
10 μ M Antimycin + 2 μ M rotenone	0	0.32 $\pm$ 0.004	77
	0.5	0.43 $\pm$ 0.03	71
	2.0	0.64 $\pm$ 0.05	56
1 mM KCN	0	0.09 $\pm$ 0.01	94
	0.5	0.1 $\pm$ 0.001	93
	2.0	0.11 $\pm$ 0.01	92

g of rat heart tissue reported by Veerkamp and co-workers [11,25,26]. Antimycin plus rotenone or KCN, which indirectly inhibit mitochondrial β -oxidation by inhibiting respiration, caused decreases in palmitate oxidation of 54% and 31% respectively (see Table 1). Inhibitors of mitochondrial fatty acid uptake, e.g. 2-tetradecylglycidic acid, 2-bromopalmitic acid and its CoA derivative, also caused only partial inhibitions of palmitate oxidation. The maximal inhibition of 87% was observed in the presence of 120 μ M 2-bromopalmitic acid. However, at a relatively high concentration of this acid, competition between inhibitor and substrate for activation by palmitoyl-CoA synthase may limit both mitochondrial and peroxisomal β -oxidation. In an attempt to evaluate the effectiveness of KCN or antimycin plus rotenone as inhibitors of mitochondrial β -oxidation, coupled rat heart mitochondria were isolated and used. Since palmitoyl-CoA synthase is inactivated by Nagarse during the preparation of coupled rat heart mitochondria [27], palmitoylcarnitine was used as a substrate and all exogenous cofactors were omitted. Only the effect of L-carnitine on the rate of β -oxidation in the absence or presence of respiratory inhibitors was evaluated. The formation of acid-soluble products from [1- 14 C]palmitoylcarnitine by rat heart mitochondria was linear for at least 6 min (see Figure 1b), and hence measurements were routinely terminated after 6 min of incubation. Since the rate of CO_2 formation was very low under the incubation conditions used in this study [28], only acid-soluble products were quantified. The formation of acid-soluble products was virtually unaffected by addition of either 0.5 mM or 2 mM L-carnitine (see Table 2). The inhibition by KCN was almost complete (94%) and virtually unaffected by addition of L-carnitine. Surprisingly, even with well-coupled rat heart mitochondria, the inhibition of β -oxidation by antimycin plus rotenone was incomplete. Moreover, the inhibition was partially relieved by addition of L-carnitine. As shown in Table 2, the inhibition caused by antimycin plus rotenone declined from 77% to 56% as the concentration of L-carnitine was increased from 0 to 2 mM. Although the reason for the incomplete inhibition and the carnitine-dependent relief of the inhibition of β -oxidation by antimycin plus rotenone has not been elucidated, it seems possible that the residual oxygen consumption in the presence of inhibitors, of 8.5 ng-atoms/min per mg of mitochondrial protein (results not shown), may sustain the remaining β -oxidation. The stimulation of β -oxidation in the presence of carnitine could be due to the efflux of acetylcarnitine from the mitochondrial matrix, which would result in the regeneration of intramitochondrial CoA in the absence of an active tricarboxylic acid cycle.

Estimation of peroxisomal β -oxidation by measuring acyl-CoA oxidase activity

Acyl-CoA oxidase is assumed to catalyse the rate-limiting step in peroxisomal β -oxidation [29,30], and if so, the activity of this enzyme is a measure of the maximal capacity of the pathway. The acyl-CoA oxidase-catalysed conversion of 4-decenoyl-CoA into 2,4-decadienoyl-CoA, which can be measured spectrophotometrically at 300 nm [22], was studied with the aim of assessing the suitability of this method for obtaining reliable values of acyl-CoA oxidase activity in tissue homogenates. The spectral changes associated with the dehydrogenation of 4-*cis*-decenoyl-CoA by purified acyl-CoA oxidase from rat liver are illustrated in Figure 2. The time-dependent emergence of an absorbance band centred around 300 nm agrees with the formation of 2,4-decadienoyl-CoA [31]. The formation of 2,4-decadienoyl-CoA was also confirmed by h.p.l.c. (results not

Figure 2 Spectral changes associated with the dehydrogenation of 4-*cis*-decenoyl-CoA by rat liver acyl-CoA oxidase

Spectrum 1 was recorded before addition of acyl-CoA oxidase; spectra 2–6 were recorded 0.5, 3, 6, 10 and 20 min respectively after addition of enzyme.

Figure 3 Activity of acyl-CoA oxidase in a rat liver homogenate

(a) Change in A_{300} as a function of time. (b) Product formation as a function of protein concentration. Acyl-CoA oxidase was assayed by use of the direct assay described in the Materials and methods section with 50 μ M 4-*cis*-decenoyl-CoA (●) or 50 μ M 4-*trans*-decenoyl-CoA (▲) as substrate.

shown). Both 4-*cis*-decenoyl-CoA and 4-*trans*-decenoyl-CoA were tested for their suitability as substrates. The absorbance increase observed with 4-*trans*-decenoyl-CoA was 4 times the change detected with the *cis* isomer (see Figure 3a). With both substrates, the increase in absorbance was linear for at least 10 min, and the rate of product formation was linearly dependent on the protein concentration up to at least 0.24 mg of rat liver homogenate (see Figure 3b). Since Hovik and Osmundsen [32] recently reported a severe inhibition of rat liver acyl-CoA oxidase by 4-*cis*-decenoyl-CoA at concentrations above 10 μ M, the kinetic parameters ($V_{\max}$, K_m) of this enzyme with 4-*trans*-decenoyl-CoA and 4-*cis*-decenoyl-CoA as substrates were determined. Figure 4 gives the results obtained with 4-*trans*-decenoyl-CoA, which do not show substrate inhibition at concentrations up to 50 μ M. Similar results (not shown) were obtained with 4-*cis*-decenoyl-CoA. In two separate experiments K_m values of 15 μ M and 13 μ M were obtained for 4-*trans*-decenoyl-CoA, whereas values for 4-*cis*-decenoyl-CoA were 22 μ M and 20 μ M. These values are similar to the K_m value of 20 μ M reported by Osumi et al. [13] for decanoyl-CoA, but are much higher than the value of 1.7 μ M determined by Hovik and Osmundsen [32] for 4-*cis*-decenoyl-CoA. In contrast, $V_{\max}$ values

Figure 4 Specific activity of purified acyl-CoA oxidase from rat liver as a function of the 4-*trans*-decenoyl-CoA concentration

Circles represent experimental values, and the line was obtained by non-linear curve fitting to the Michaelis-Menten equation by use of the Sigma plot program.

Figure 5 Evaluation of the effect of Triton X-100 on the activity of acyl-CoA oxidase and assessment of the stability of 2-*trans*-4-*trans*-decadienoyl-CoA

(a) Acyl-CoA oxidase activity present in a rat liver homogenate was measured with 50 μ M 4-*trans*-decenoyl-CoA (▲) and 50 μ M 4-*cis*-decenoyl-CoA (●) as a function of the Triton X-100 concentration. (b) The A_{300} of 2.5 μ M (▲) and 5 μ M 2-*trans*-4-*trans*-decadienoyl-CoA (▼) was determined in the presence of rat liver homogenate (40 μ g/ml) as a function of time.

obtained with 4-*cis*-decenoyl-CoA in two separate experiments were 0.75 and 0.79 unit/mg of protein, or 12.5 and 13.2 μ mol/s per g of protein, and thus were similar to the activity of 11 ± 3 μ mol/s per g of protein reported by Hovik and Osmundsen [32]. With 4-*trans*-decenoyl-CoA, $V_{\max}$ values of 4.1 and 3.6 units/mg of protein were observed.

In order to use this assay procedure for measuring the activity of acyl-CoA oxidase in tissue homogenates, it was important to evaluate how a detergent such as Triton X-100 affects the activity of the enzyme, and if the reaction product 2,4-decadienoyl-CoA is stable under the assay conditions. The data shown in Figure 5(a) demonstrate a moderate decline of the acyl-CoA oxidase activity with increasing Triton X-100 concentration. With 4-*trans*-decenoyl-CoA as substrate and at a typical assay concentration of 0.05% Triton X-100, the activity of acyl-CoA oxidase was 6% lower in the presence than in the absence of detergent. The addition of product instead of substrate to a standard assay mixture resulted in a moderate (9%) decrease in

Table 3 Activities of acyl-CoA oxidase in extracts of several rat tissues and of the purified rat liver enzyme

Acyl-CoA oxidase was assayed by the direct method as described in the Materials and methods section and by the coupled method of Small et al. [19]. Substrates at concentrations of 50 μ M were 4-*trans*-decenoyl-CoA (4trC₁₀) and decanoyl-CoA (C₁₀). Other abbreviation: ND, not detectable. Values are means of at least three measurements, with S.D. less than 10% of the mean value.

Enzyme source	Substrate ...	Acyl-CoA oxidase activity (nmol/min per mg of protein)		
		Direct assay 4trC ₁₀	Coupled assay	
			4trC ₁₀	C ₁₀
Liver homogenate		5.9	0.97	2.1
Heart extract		0.49	0.09	0.18
Muscle extract		≤ 0.13	ND	ND
Kidney homogenate		2.2	0.42	0.86
Brain extract		≤ 0.16	≤ 0.06	≤ 0.05
Purified acyl-CoA oxidase		2120	234	595

absorbance over a 10 min period (see Figure 5b). A concern about the specificity of this assay was based on the possibility that medium-chain acyl-CoA dehydrogenase, which is known to act on 4-decenoyl-CoA [33], may contribute to the formation of product. When acyl-CoA dehydrogenase was assayed with 4-*trans*-decenoyl-CoA as substrate and phenazine methosulphate as electron acceptor, by a procedure that is similar to a specific assay of medium-chain acyl-CoA dehydrogenase [34], activities of 6.5 and 26 m-units/mg of protein were measured in homogenates of skeletal muscle and brain respectively. Since acyl-CoA oxidase was virtually undetectable in these homogenates (see Table 3), acyl-CoA dehydrogenases do not make a significant contribution to the formation of product under conditions used to assay acyl-CoA oxidase.

Activities of acyl-CoA oxidase in homogenates of several rat tissues were determined by the direct measurement of product formation and by the coupled assay as detailed by Small et al. [19]. The results shown in Table 3 demonstrate that activities of this enzyme based on the direct assay are several-fold higher than activities obtained by use of the coupled assay. Moreover, when a stoichiometry of 3.5 mol of 2,7-dichlorofluorescein oxidized/mol of NAD⁺ reduced [19] is taken into account, the activities determined by the direct assay in tissue homogenates or extracts were approx. 20 times the activities obtained by the coupled assay with the same substrate. Activities were also determined with decanoyl-CoA as substrate to aid in efforts to estimate rates of peroxisomal β -oxidation. Decanoyl-CoA was chosen as a substrate because it yields an acyl-CoA oxidase activity that is close to that obtained with palmitoyl-CoA, but, unlike palmitoyl-CoA, it does not cause substrate inhibition [13]. When activities measured by one method in different tissues are compared, the two assay methods yield comparable results. The activity in a liver homogenate was about 2.5 times that in a kidney homogenate and at least 10 times that in a heart extract. Activities in skeletal muscle and brain were hardly or not detectable. Activities were also measured by an assay based on the substrate-dependent formation of NADH (Method 3). This assay should yield rates that are similar to rates of peroxisomal β -oxidation, if acyl-CoA oxidase catalyses the rate-limiting step of the pathway. Activities measured by this method with decanoyl-CoA as substrate were 3.7 nmol/min per mg of protein with a rat liver homogenate and

1140 nmol/min per mg of protein for the purified rat liver oxidase.

The specific activity of acyl-CoA oxidase in a heart extract as measured by the coupled assay was 0.11 nmol/min per mg of protein with palmitoyl-CoA as substrate, and thus was 20% higher than the activity observed with 4-*trans*-decenoyl-CoA (see Table 3). Assuming that this activity ratio also applies to the direct assay, the best estimate of palmitoyl-CoA oxidase activity in a heart extract is 0.6 nmol/min per mg of protein, or 0.3 nmol/min per mg of protein in a heart homogenate which contains twice as much protein as the extract.

DISCUSSION

A commonly used approach for estimating the contribution of peroxisomal β -oxidation to the total fatty acid oxidation by a tissue is based on the inhibition of mitochondrial β -oxidation by inhibitors of respiration or fatty acid uptake. Fatty acid oxidation, which is unaffected by mitochondrial inhibitors, is assumed to represent β -oxidation in peroxisomes. However, if the inhibition of mitochondrial β -oxidation is incomplete, peroxisomal β -oxidation would be overestimated. This problem would be most serious with tissues that have a low capacity for peroxisomal β -oxidation compared with mitochondrial oxidation. Based on the data presented in Table 1, the contribution of peroxisomal β -oxidation to the total palmitate oxidation could be as high as 70%. This value is much higher than values between 20% and 30% estimated by Veerkamp and van Moerkerk [11], and it seems an unlikely value, in view of the fact that heart cells contain many mitochondria but few and only small peroxisomes [8]. A major problem with these measurements is the damage that occurs to subcellular organelles during tissue homogenization. This is especially a problem when tougher tissues such as heart are homogenized. Veerkamp et al. [26] reported that 16% of citrate synthase, which is a marker enzyme for the mitochondrial matrix, was released into the 20000 g supernatant during the preparation of rat heart homogenates. If citrate synthase is released from mitochondria, enzymes of β -oxidation will also escape and act on L-3-hydroxyacyl-CoAs formed by partial β -oxidation of fatty acids in mitochondria, or even act on fatty acyl-CoAs in the presence of CoA plus NAD⁺ and some artifactual system capable of reoxidizing acyl-CoA dehydrogenase. Clearly, attempts to estimate peroxisomal β -oxidation by inhibiting the mitochondrial pathway are fraught with problems, and most likely will not yield satisfactory results, especially when tough tissues are studied that contain many mitochondria but few peroxisomes.

A better way of estimating the activity of peroxisomal β -oxidation is by measuring the activity of acyl-CoA oxidase, which is thought to catalyse the rate-limiting reaction of this pathway [29,30]. Acyl-CoA oxidase is most commonly assayed by a procedure in which the formation of H₂O₂ is coupled to the oxidation of a dye that can be measured spectrophotometrically. The problem associated with such assays is that only a fraction of H₂O₂ may react with the dye, due to its instability and due to competing reactions. In contrast, the assay introduced by Hovik and Osmundsen [22], and further evaluated by us, relies on the direct spectrophotometric measurement of product formation. Since 2,4-decadienoyl-CoA formed by the oxidase is relatively stable, even in the presence of tissue extracts, this method seems to yield reliable values of acyl-CoA oxidase with 4-decenoyl-CoA as substrate. Not surprisingly, activities obtained by the direct method are much higher than activities measured by the coupled assay of Small et al. [19]. However, the relative activities obtained by the direct and coupled assays with different tissue

homogenates are comparable. Thus it seems justifiable to use relative activities measured by the coupled assay with different substrates for estimating oxidase activities based on the direct assay with 4-*trans*-decenoyl-CoA as substrate. For example, since the oxidase is twice as active with decanoyl-CoA as it is with 4-*trans*-decenoyl-CoA (see Table 3), the true decanoyl-CoA oxidase activity in a rat liver homogenate is estimated to be close to 12 nmol/min per mg of protein. This activity is still 3 times the activity of 3.7 nmol/min per mg of protein obtained by decanoyl-CoA-dependent reduction of NAD⁺ in an assay based on the oxidase-initiated β -oxidation. Obviously, the substrate-dependent reduction of NAD⁺ yields more realistic activities of acyl-CoA oxidase than does the assay by Small et al. [19], but still underestimates the maximal activity of acyl-CoA oxidase. However, in a study like this, aimed at estimating peroxisomal β -oxidation, the substrate-dependent NAD⁺ reduction may yield the more realistic activities because inhibitory effects of intermediates and cofactors, as reported for CoA [32], are reflected in the measured activities.

A comparison of the specific activity of 0.3 nmol/min per mg of protein estimated for palmitoyl-CoA oxidase with an activity of 7.7 nmol/min per mg of protein determined for total palmitate β -oxidation prompts the conclusion that the peroxisomal pathway makes only a minor contribution (4%) to total palmitate oxidation in a rat heart homogenate. If the lower oxidase activity based on the substrate-dependent NAD⁺ reduction is considered, the contribution of peroxisomes to palmitate β -oxidation seems to be insignificant. However, peroxisomal β -oxidation would be the major pathway and therefore essential for the degradation of fatty acids, e.g. very-long-chain fatty acids, that are poorly or not at all oxidized by mitochondria [7].

This investigation was supported in part by U.S. Public Health Service Grants HL18089 and HL30487 of the National Heart, Lung, and Blood Institute, and by Grant RR03060 to Research Centers of Minority Institutions.

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Received 4 February 1994/31 March 1994; accepted 13 April 1994