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Studies on the peroxisomal oxidation of palmitate and lignocerate in rat liver

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We have investigated the pathways involved in the peroxisomal oxidation of palmitate and lignocerate, measured as the cyanide-insensitive formation of acetyl units, in rat-liver homogenates. The peroxisomal β-oxidation of both fatty acids is dependent on the presence of ATP, coenzyme A, NAD^+ and Mg^{2+}. However, there is a striking difference in the dependence of the rate of oxidation of the two substrates on the concentration of the individual cofactors, especially ATP. The peroxisomal β-oxidation of lignocerate was inhibited to a progressively greater extent by increasing concentrations of palmitate and vice versa. Activation of lignoceric acid to lignoceroyl-CoA, however, was not inhibited by increasing concentrations of palmitate, and vice versa. It can be concluded that the peroxisomal palmitate and lignocerate β-oxidation pathways differ in at least one enzymic reaction (the synthetase), but that the two pathways share at least one common step.

Introduction

Fatty acid β-oxidation was long thought to be the prerogative of mitochondria. Following the discovery by Cooper and Beevers [1] that castor bean endosperm glyoxysomes, organelles closely related to peroxisomes, contain a fatty acid β-oxidation system, fatty acyl-CoA β-oxidation activity was demonstrated in peroxisomes from rat liver [2] and from various other mammalian tissues (for reviews see Refs. 3–5). It was shown by Lazarow [6] that fatty acyl-CoA oxidation in rat-liver peroxisomes proceeds via a true β-oxidation process through successive steps of dehydrogenation, hydration, dehydrogenation and thiolytic cleavage. Like mitochondria and microsomes, peroxisomes contain an ATP-dependent long-chain fatty acid-activating enzyme [7–9] which appears to be located at the cytoplasmic side of the organelle [9]. There is considerable evidence that the same long-chain acyl-CoA synthetase is present in mitochondria, microsomes and peroxisomes [10].

Despite these apparent similarities in architecture between the β-oxidation systems in peroxisomes and mitochondria, there are several important differences between the two organelles with regard to the properties of the individual enzymes, cofactor requirements, coupling to energy production and specificity for fatty acids of different chain lengths [3–5]. Since acyl-CoA

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oxidase, at least from rat liver, is only reactive towards acyl-CoA esters having six or more carbon atoms in their acyl moiety, peroxisomes are incapable of oxidizing fatty acids to completion [3–5]. Instead, as originally suggested by Bremer [11] fatty acids are chain-shortened to products with fewer carbon atoms which are further degraded in the mitochondria. Although peroxisomes are capable of oxidizing a wide variety of fatty acids (see for example Ref. 12), they seem particularly well suited to catalyze the chain-shortening of monounsaturated long-chain fatty acids such as erucic acid (22:1) (for review see Ref. 13) and, especially, of very-long-chain fatty acids such as lignoceric acid (24:0) and cerotic acid (26:0) [14,15]. Indeed, very-long-chain fatty acids accumulate in tissues and body fluids from patients with the Zellweger syndrome [16] in which peroxisomes are known to be absent.

The question arises of whether the β-oxidation of different fatty acid substrates in peroxisomes follows the same pathway or not. We have therefore compared the peroxisomal β-oxidation of palmitate, a long-chain fatty acid (16:0) and lignocerate, a very-long-chain fatty acid (24:0) in rat-liver homogenates. The results of this study show that at least one step in the β-oxidation of the two substrates must be different, whereas on the other hand the two pathways have at least one step in common.

Materials and Methods

Peroxisomal fatty acid oxidation was measured essentially as described by Mannaerts et al. [17] using a modification of the method described by Singh and Kishimoto [18] to solubilize the fatty acids. Incubations were carried out at 37 °C using a medium of the following composition: 300 mM sucrose, 50 mM morpholinopropanesulphonic acid (Mops; adjusted to pH 7.4 with NaOH), 10 mM ATP, 1 mM NAD^+, 100 μM FAD, 5 mM $MgCl_2$, 200 μM coenzyme A, 5 mM KCN and 10 μM 1-^{14}C-labelled palmitate or lignocerate dissolved in α-cyclodextrin (final concentration of α-cyclodextrin in the reaction mixture 1 mg/ml). The fatty acid substrates were prepared as 100 μM stock solutions by the following procedure: [1-^{14}C]palmitic acid or [1-^{14}C]lignoceric acid dissolved in either benzene or toluene was taken to dryness in a glass tube. This was followed by the addition of 100 mM Tris-HCl (pH 8.5) containing 10 mg/ml α-cyclodextrin. Full solubilization was obtained via a 30 min incubation in a sonicating water bath at room temperature. Reactions were started by the addition of rat-liver homogenate (20 μl) in 250 mM sucrose plus 2 mM Mops-NaOH (pH 7.4) at final protein concentrations of 1 mg/ml in the case of lignocerate and 0.2 mg/ml in the case of palmitate oxidation. Reactions were terminated as described before [19] by transferring a 150 μl portion of the incubation mixture to a glass tube containing 3.25 ml of methanol/chloroform/heptane (1.41:1.25:1.00, v/v). Samples were vigorously shaken and subsequently mixed with 1.05 ml of 0.1 M sodium acetate (pH 4.0) as described by Krisans et al. [8]. After vigorously shaking for 5 min, samples were centrifuged at 4 °C for 10 min at $700 \times g_{av}$. Control experiments carried out with [^{14}C]palmitate, [^{14}C]lignocerate, [^{14}C]acetyl-CoA and [^{14}C]acetate revealed that 0.1%, 0.1%, 100% and 93.0%, respectively, of the compounds was recovered in the upper, aqueous phase. In order to remove [^{14}C]palmitoyl-CoA and [^{14}C]lignoceroyl-CoA from the upper phase, 1.5 ml of the upper, aqueous phase was added to 0.3 ml 2 N NaOH and incubated at 50 °C for 45 min to hydrolyze all CoA esters. The resulting mixture was subsequently acidified by addition of 60 μl concentrated H_2SO_4. Extraction of [^{14}C]palmitic acid and [^{14}C]lignoceric acid was carried out by adding 1.5 ml of an artificially prepared lower, organic phase. This procedure was repeated once and the radioactivity remaining in the supernatant was determined. The radioactive material in the supernatant represented acetic acid, as shown by gas chromatography and paper chromatography on Whatmann 3 MM paper according to Krisans et al. [8].

The activity of palmitoyl-CoA synthetase and lignoceroyl-CoA synthetase was measured at 37 °C using a medium of the following composition: 150 mM Tris-HCl (pH 8.5) 200 μM coenzyme A, 10 mM ATP, 10 mM $MgCl_2$ and 10 μM [1-^{14}C]palmitate or lignocerate dissolved in α-cyclodextrin (see above). Reactions were started by adding 20 μl rat-liver homogenate (final volume 200 μl) at protein concentrations of 50–100 μg/ml

in the case of lignocerate and 1–2 μg/ml in the case of palmitate. After 10 min reactions were terminated by transferring a 150 μl portion of the incubation mixture to a glass tube containing 750 μl Dole's reagent. After vigorous mixing, protein was removed by centrifugation and 650 μl of the supernatant were added to 350 μl heptane plus 190 μl of a solution containing 400 mM Mops-NaOH (pH 6.5). The lower (aqueous) layer was washed three times with artificially prepared upper phase and the radioactivity in the final lower layer measured.

Rat-liver homogenates were prepared by gentle homogenization of the liver in 10 vol. of a solution containing 300 mM sucrose plus 2 mM Mops-NaOH (pH 7.4), using a Dounce homogenizer. The homogenate was centrifuged for 5 min at 6500 rpm in a Sorvall-RC centrifuge (SS rotor type SS34) to remove unbroken cells and nuclei. The supernatant was kept on ice until use.

Results and Discussion

Table I shows that when rat-liver homogenates are incubated in the presence of 5 mM KCN to block the mitochondrial β-oxidation system, the oxidation of lignocerate, like that of palmitate [17], is dependent upon the presence of ATP, coenzyme A, Mg^{2+} and NAD^+. As shown in Fig. 1 striking differences were observed with regard to the dependency of the rate of oxidation of palmitate and lignocerate on the concentration of these cofactors. This was especially apparent in the case of ATP; the concentration required to give half maximal rates of β-oxidation was 9.6 mM in case of lignocerate and 0.11 mM in case of palmitate.

TABLE I

COFACTOR REQUIREMENTS FOR THE PEROXISOMAL β-OXIDATION OF PALMITATE AND LIGNOCERATE IN RAT-LIVER HOMOGENATES

Peroxisomal fatty acid β-oxidation was measured as described in the text using palmitate and lignocerate as substrates. Values are means ± S.D. of 2–4 different experiments. The rates of oxidation in the complete system were 49.2 ± 8.1 ($n = 4$) pmol/min per mg protein for palmitate and 22.6 ± 3.2 ($n = 4$) pmol/min per mg protein for lignocerate.

Omission	Activity (% of maximum)	
	palmitate	lignocerate
None	100	100
ATP	1	1
Coenzyme A	2	2
$MgCl_2$	17	13
NAD^+	4	5
FAD^+	96	100

In order to obtain information about possible common steps in the peroxisomal β-oxidation of lignocerate and palmitate, competition experiments were carried out. Fig. 2A shows that the rate of formation of [1-^{14}C]lignoceroyl-CoA was not influenced by addition of increasing concentrations of unlabelled palmitate. In contrast, the rate of the peroxisomal β-oxidation of lignocerate, measured as the cyanide-insensitive formation of [1-^{14}C]acetyl units, was inhibited to a progressively greater extent by increasing concentrations of unlabelled palmitate. Similar results were obtained with palmitate: [1-^{14}C]palmitate activation to [1-^{14}C]palmitoyl-CoA was not influenced by addition of unlabelled lignocerate, whereas oxidation of [1-^{14}C]palmitate to [1-^{14}C]acetyl units was inhibited by unlabelled lignocerate (Fig. 2B). It is remarkable that palmitate did not have a stronger inhibitory effect on [1-^{14}C]lignoceric acid β-oxidation as compared to the inhibition of [1-^{14}C]palmitic acid β-oxidation by lignocerate, since palmitoyl-CoA levels will be much higher due to the high rate of palmitate activation as compared to lignocerate activation. This finding suggests a preference of the peroxisomal β-oxidation system for lignoceroyl-CoA.

The results of Fig. 2 provide direct evidence for the involvement of different enzymes in the formation of lignoceroyl-CoA and palmitoyl-CoA, respectively. Furthermore, although the activation of palmitate and lignocerate is catalyzed by different enzymes, further catabolism of the two substrates via peroxisomal β-oxidation must involve at least one common step.

Bhusnan et al. [20] have brought forward evidence indicating that in rat-brain microsomes the synthesis of lignoceroyl-CoA and palmitoyl-CoA from lignocerate and palmitate, respectively, is brought about by separate proteins. Kishimoto and co-workers [21,22], on the other hand, suggest that both in rat-liver and rat-brain microsomes a single enzyme is responsible for the activation of

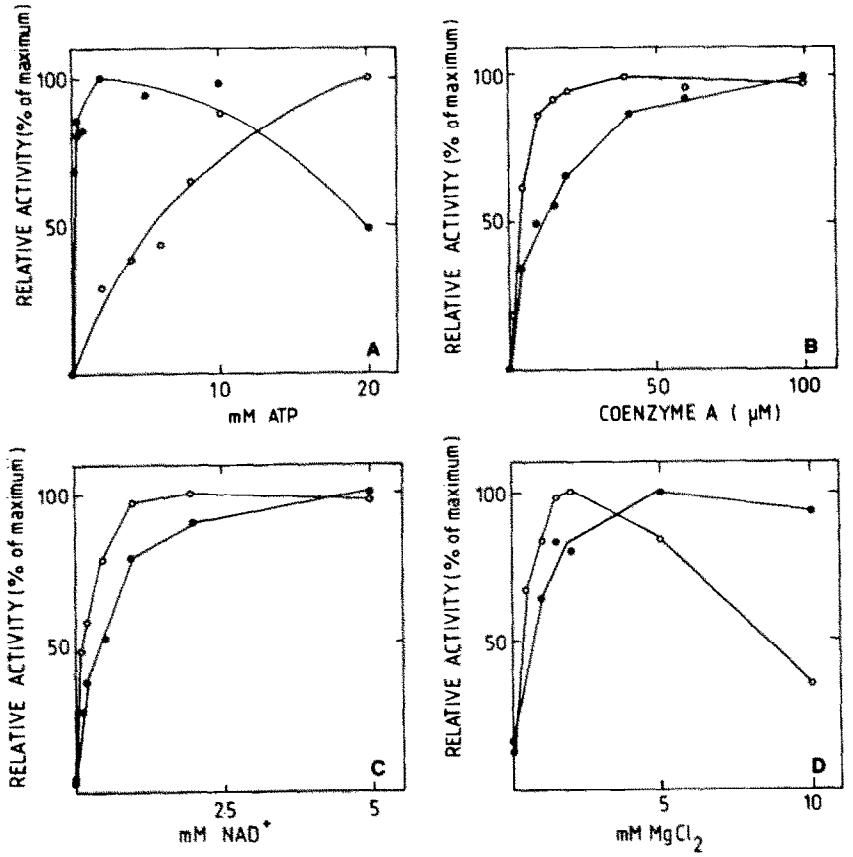

Fig. 1. Dependence on the concentration of cofactors of the peroxisomal oxidation of [1-^{14}C]lignocerate (○) and [1-^{14}C]palmitate (●) in rat-liver homogenates. The cyanide-insensitive generation of acetyl units from lignocerate and palmitate was studied as described in the text. When one of the cofactors was omitted, the others were kept constant at their standard concentrations (see text). Exactly analogous results were obtained in another experiment.

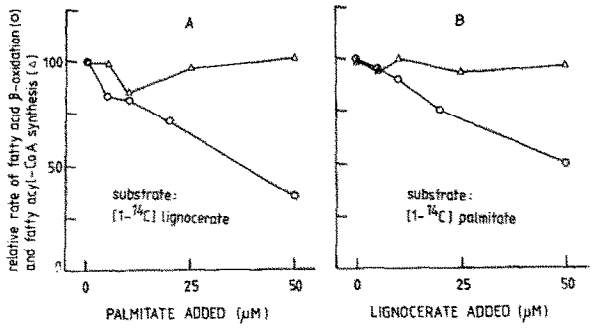

Fig. 2. Effect of different concentrations of unlabelled palmitate and lignocerate on the activation and peroxisomal β-oxidation of [1-^{14}C]lignocerate and [1-^{14}C]palmitate, respectively, in rat-liver homogenates. Peroxisomal [1-^{14}C]lignocerate and [1-^{14}C]palmitate oxidation and the activities of lignoceroyl-CoA synthetase and palmitoyl-CoA synthetase were measured as described in the text. In A the substrate was [1-^{14}C]lignocerate and different concentrations of unlabelled palmitate were added. In B the substrate was [1-^{14}C]palmitate and different concentrations of unlabelled lignocerate were added. Similar results were obtained in three other experiments. The rates of fatty acid activation and fatty acid β-oxidation were: palmitate oxidation, 45.1 pmol/min per mg protein; palmitate activation, 29.1 nmol/min per mg protein; lignocerate oxidation, 21.6 pmol/min per mg protein; lignocerate activation, 481 pmol/min per mg protein.

palmitate and lignocerate, the substrate specificity being dependent upon the aggregation state of the enzyme as determined by its lipid micro-environment. The relationship between the activities we have measured in rat-liver homogenates and those described by Bhusnan et al. [20] in rat-brain microsomes is not clear at present.

The long-chain fatty acyl-CoA synthetase which

is most active towards fatty acids with a chain length of 10–18 carbons (for reviews see Refs. 5 and 23) is present in mitochondria, microsomes and peroxisomes in rat liver [10]. We have carried out preliminary experiments on the distribution of lignoceroyl-CoA synthetase activity in rat liver. The results indicate that this activity is present in microsomes and peroxisomes but not in mitochondria. Furthermore, the ratio palmitoyl-CoA synthetase to lignoceroyl-CoA synthetase differs in the two fractions. These data thus provide further evidence that the two synthetase activities are, indeed, due to different enzymes.

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