PEROXISOMAL OXIDATION OF LONG CHAIN FATTY ACIDS

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1. Introduction

Peroxisomes isolated from rat liver have been shown to β-oxidize fatty acids [1,2]. The hypolipidaemic drug clofibrate (α-*p*-chloro-phenoxy-isobutyrate) induces proliferation of liver peroxisomes [3] and mitochondria [4], and stimulates peroxisomal oxidation of palmitate [5]. The metabolic role of peroxisomal fatty acid oxidation is not clear, although it has been claimed that peroxisomes may be responsible for a major portion of the oxidation of palmitate normally attributed to mitochondria [2].

A marked increase in the ability to oxidise fatty acids, in particular very long chain fatty acids, e.g., erucic acid, has been shown in hepatocytes isolated from rats fed clofibrate [6]. It is also clear that mitochondrial fatty acid oxidation is stimulated by clofibrate feeding. This effect is primarily expressed with fatty acids shorter than palmitate, and there is only a marginal stimulatory effect on oxidation of erucic acid [6]. These findings, suggest that extra-mitochondrial sites for oxidation of erucic acid are present in the rat liver.

We have investigated the ability of isolated peroxisomes to oxidize fatty acids of various chain lengths, and attempted to identify some oxidation products from peroxisomal oxidation of [14-^{14}C]erucoyl-CoA. A recent finding that NADP stimulates chain-shortening of [14-^{14}C]erucic acid by mitochondrial preparations [7], led us to examine the effect of NAD and NADP on peroxisomal oxidation of [14-^{14}C]erucoyl-CoA.

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2. Materials and methods

Brassidic acid (*trans*-$C_{22:1\ (9)}$), elaidic acid (*trans*-$C_{18:1\ (9)}$), gadeoleic acid (*cis*-$C_{20:1\ (11)}$) and erucic acid (*cis*-$C_{22:1\ (9)}$) were purchased from Nu-Chek Preps., Elysian, MN. All other fatty acids were purchased from E. Merck, Darmstadt. Coenzyme A (grade 1-L), Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) and EGTA were purchased from Sigma Chemical Co., St Louis, MO. [14-^{14}C]Erucic acid was purchased from CEA, Gif-sur-Yvette. All other reagents were of analytical grade, or highest purity available.

Liver peroxisomes were prepared from male albino Wistar rats (200–250 g), which had been fed a standard pelleted diet containing 0.3% (w/w) clofibrate for 2–3 weeks. Livers were homogenised in a medium containing 300 mM mannitol, 10 mM Hepes, 1 mM EGTA and 0.1% (v/v) ethanol (pH 7.2). From this a fraction similar to the L-fraction in [8] was prepared. About 1 ml (80 mg protein) of this fraction was layered on the top of a 42–51% (w/w) sucrose gradient (gradient vol. 30 ml). This was centrifuged at 54 300 × g_{av} for 30 min in a Sorvall OTD-65 ultracentrifuge, using a TV-850 vertical rotor. The pooled peroxisomal fractions were diluted with 3 vol. 5 mM Hepes (pH 7.4). The peroxisomes were subsequently sedimented at 32 000 × g_{av} for 15 min by using the SA-600 centrifuge head. The resulting pellet was resuspended in a minimal volume of homogenisation medium, giving a final 20–30 mg protein/ml. These peroxisomal preparations contained 24% of the uricase (EC 1.7.3.3) activity present in the starting homogenate. The cytochrome *c* oxidase (EC 1.9.3.1) activity (assayed in the presence of 0.02% (v/v) of Triton X-100) was only 0.2% of

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that present in the starting homogenate. Contamination from endoplasmic reticulum was similarly about 1% (measured as rotenone-insensitive NADPH cytochrome *c* reductase (EC 1.6.2.3), and about 6% with respect to lysosomes (measured as β-*N*-acetyl-D-glucoseaminidase activity (EC 3.2.1.29)). All peroxisomal preparations were used within 4 h of the animal being killed.

Peroxisomal fatty acid oxidation was measured as in [2], with the exception that bovine serum albumin was absent from the assay. The assay contained 0.5 mM NAD, 0.2 mM CoA, 0.5 mM dithiothreitol and 0.005% (v/v) Triton X-100. Rates of NAD reduction were measured by using an Aminco DW-2 spectrophotometer operated in dual wavelength mode (340–400 nm). Assays were carried out at 36°C.

Peroxisomal oxidation of [14-^{14}C]erucoyl-CoA was also measured as release of $HClO_4$ (5%, v/v) soluble radioactivity. The incubation mixture contained 68 mM KCl, 10 mM Hepes, 10 mM P_i, 0.15 mM CoA, 1 mM dithiothreitol, 20 μM [14-^{14}C]erucoyl-CoA (spec. act. 3904 dpm/nmol) and defatted bovine serum albumin (30 μg/nmol acyl-CoA) (pH 7.4). Samples taken from these incubations were also used for radio-gas–liquid chromatographic analysis. Incubations were carried out at 30°C.

[14-^{14}C]Erucoyl-CoA was prepared from the corresponding succinimide ester essentially as in [9]. Unlabelled acyl-CoA ester were synthesized by the mixed anhydride procedure [10]. Purities of acyl-CoA preparations were checked by thin layer chromatography [11]. Radio-gas–liquid chromatographic analysis showed that [14-^{14}C]erucoyl-CoA contained only one peak of radioactivity.

The fatty acid products from peroxisomal oxidation of [14-^{14}C]erucoyl-CoA were analysed as methyl esters by radio-gas–liquid chromatography on a column of 10% SP-2340 on Chromosorb W AW (Supelco Inc., PA) as in [6].

Solutions of acyl-CoA esters were assayed spectrophotometrically [12] by using purified carnitine palmitoyltransferase (EC 2.3.1.21) (H.O., unpublished), or carnitine acetyltransferase (EC 2.3.1.7). Behenoyl-CoA ($C_{22:0}$) had to be assayed as the hydroxamate ester [14], because it was not a good substrate for carnitine palmitoyltransferase.

Student's paired *t*-test was used to determine the significance of differences between means. Proteins were measured by using the Bio-Rad protein assay kit (Bio-Rad Labs, USA).

3. Results and discussion

It has been shown that peroxisomal rates of oxidation of short chain acyl-CoA esters are very low; butyryl-CoA is not oxidized at all [2]. Maximal rates of oxidation were found with lauroyl-CoA and palmitoyl-CoA [2]. The data presented in fig.1 partly confirm these findings. We have, however, found a clear peak rate of oxidation with myristoyl-CoA, as far as the saturated acyl-CoA esters are concerned. With carbon chain lengths >16 atoms a marked decrease in oxidation rate is observed. This suggests that peroxisomes are not likely to be involved in the oxidation of long saturated acids. With mono-unsaturated acyl-CoA esters of identical chain lengths (palmitoleoyl-CoA, oleoyl-CoA, gadeoleoyl-CoA and erucoyl-CoA), however, rates of oxidation were higher by a factor of 10 (excepting palmitoleoyl-CoA). In this series a clear peak rate of oxidation was achieved with oleoyl-CoA. These results therefore suggest that peroxisomes, in clofibrate-treated rats, may play a significant role in the cellular oxidation of long mono-unsaturated fatty acids, in particular the $C_{22:1}$-monoenes (here represented by erucoyl-CoA).

The peroxisomal rate of erucoyl-CoA oxidation may be taken to be equivalent to ~10 nmol acetyl-groups/min/mg protein (assuming 1 NADH generated = 1 acetyl-group produced [2]). The mitochondrial oxidation of erucoylcarnitine has been measured to ~5–10 nmol acetyl-groups/min/mg protein [6,14,15]. This suggests that the peroxisomal specific activity of erucic acid oxidation is not less than that of the mitochondria.

The difference in oxidation rates between saturated and mono-unsaturated fatty acids observed with isolated peroxisomes (fig.1) contrasts with the mitochondrial rates of β-oxidation of the corresponding substrate pairs. With isolated mitochondria only marginal differences in rates of oxidation of stearoyl-/oleoylcarnitine and aracidoyl-/gadeoleoylcarnitine have been found [14].

Another noteworthy property of peroxisomal

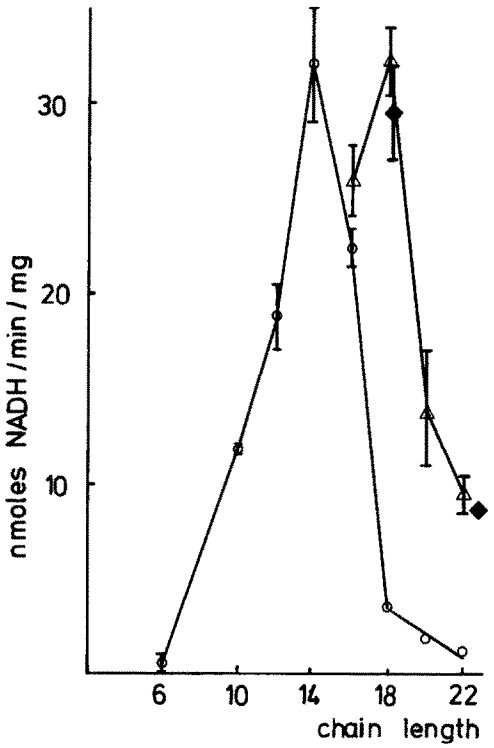

Fig.1. Rates of oxidation of saturated and mono-unsaturated acyl-CoA esters of different chain lengths by isolated peroxisomes. Rates of acyl-CoA-dependent NAD reduction have been plotted against carbon chain length of the various acyl-CoA esters. The plotted values represent the mean and SD obtained with at least 3 different peroxisomal preparations. Each assay contained 10 μM acyl-CoA and 60–150 μg peroxisomal protein, in 2 ml total vol. The details of the assay are in section 2. Results obtained with saturated acyl-CoA esters are indicated by (○) and those obtained with mono-unsaturated species by (△). The mono-unsaturated acyl-CoA esters used were: palmitoleoyl-CoA ($C_{16:1}$), oleoyl-CoA ($C_{18:1}$), gadeoyl-CoA ($C_{20:1}$) and erucoyl-CoA ($C_{22:1}$). Results obtained with the *trans*-isomers elaidoyl-CoA ($C_{18:1}$) and brassidoyl-CoA ($C_{22:1}$) are indicated by (◆).

β-oxidation is that the rate of oxidation of elaidoyl-CoA (*trans*-$C_{18:1}$) is not significantly different from that of the corresponding *cis*-isomer (oleoyl-CoA) (P=0.85, n=8; see fig.1). This is also different from mitochondrial β-oxidation, where it has been found that elaidoylcarnitine is oxidized considerably slower than oleoylcarnitine [16]. Brassidoyl-CoA (*trans*-$C_{22:1}$) is similarly oxidized by peroxisomes at a rate which is not significantly different from that of the corresponding *cis*-isomer (erucoyl-CoA). Mitochondrial oxidation of the corresponding acylcarnitines is not significantly different, but this may be due to very low rates of β-oxidation of these acylcarnitines [14]. These findings, however, suggest that peroxisomes in clofibrate-treated animals may be important in the cellular oxidation of *trans*-fatty acids.

The recorder tracing presented in fig.2 shows that a limiting amount of erucoyl-CoA undergoes about three relatively fast cycles of oxidation, as measured by NADH generated. Further oxidation is much slower, although sustained. This suggests that peroxisomal β-oxidation is primarily concerned with chain-shortening of erucoyl-CoA, although some further oxidation takes place. A similar conclusion has been reported as regards peroxisomal oxidation of palmitoyl-CoA, although the number of β-oxidation cycles has varied from 2 [1] to 5 [2]. Erucoyl-CoA was always found to be oxidized in this way, irrespective of the quantity of erucoyl-CoA added (data not shown).

Data from the literature suggest that peroxisomal β-oxidation is entirely dependent on NAD, although nothing appears to have been reported regarding

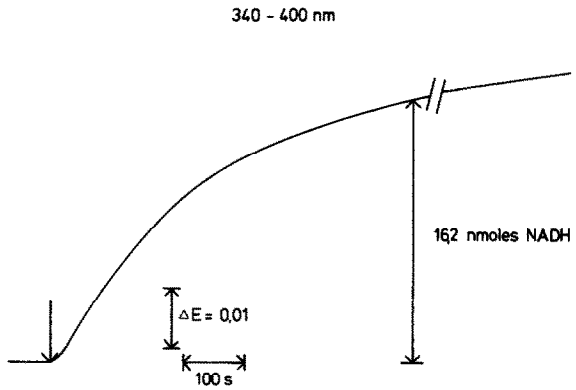

Fig.2. Progress-curve for NAD reduction caused by a limiting amount of erucoyl-CoA. A typical tracing showing that erucoyl-CoA undergoes three fast cycles of oxidation followed by a steady, but much slower, rate of reaction. The tracing shown here resulted from the addition of 5 nmol erucoyl-CoA. As 16.2 nmol NADH have been formed after 6 min; this gives 16.2/5–3.2 cycles of β-oxidation. The unlabelled arrow indicates point of addition of 5 nmol erucoyl-CoA. This assay contained 110 μg peroxisomal protein. The details of the assay have otherwise been described in the legend to fig.1

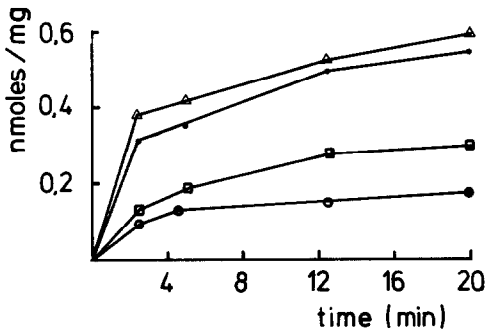

Fig.3. The effect of NAD and NADP on peroxisomal oxidation of [14-^{14}C]erucoyl-CoA. The oxidation of [14-^{14}C]erucoyl-CoA was followed by measuring the release of acid-soluble radioactivity after various times of incubation in the presence of 0.5 mM NAD alone (•), 0.5 mM NADP alone (□), in the combined presence of 0.5 mM NAD and 0.5 mM NADP (△) and in the absence of added NAD and NADP (○). The incubation mixture contained 0.55 mg peroxisomal protein/ml mixture. The details are in section 2.

NADP [1,2]. The data in fig.3 show that, although NAD alone gives practically maximal oxidation of [14-^{14}C]erucoyl-CoA, there is significant oxidation also in the presence of NADP alone. Interestingly, the combined presence of NAD and NADP gives a slightly higher extent of oxidation than NAD alone. This pattern was found with 4 different peroxisomal preparations, although the amount of radioactivity released has differed by a factor of 2. The latter phenomenon may be due to different degrees of induction of peroxisomal β-oxidation. As regards NADP, no NADP reduction was observed with the spectrophotometric assay for peroxisomal β-oxidation, indeed when NADP was added prior to NAD inhibition of NAD reduction was observed (data not shown).

The finding that mitochondrial preparations can be stimulated to shorten [14-^{14}C]erucic acid in the presence of added NADP [7] may be explained in the light of the above results. As mitochondria prepared by conventional centrifugation will contain appreciable quantities of peroxisomes, this phenomenon is likely to be due to peroxisomal oxidation. No additional sites for erucic acid oxidation need be postulated.

In the light of our finding that peroxisomal β-oxidation of erucoyl-CoA primarily involves three cycles of oxidation we have analysed the fatty acid oxidation products, and the results are presented in fig.4. It can be seen that that the intermediates generated by the first three cycles of oxidation constitute ~90% of detectable chain-shortened products, in agreement with the spectrophotometric results (fig.3). A small amount of $C_{14:1}$ (product of the fourth oxidation cycle) was also found. Samples were also examined for shorter oxidation products (e.g., acetate, as volatile fatty acids), but no detectable amounts were found.

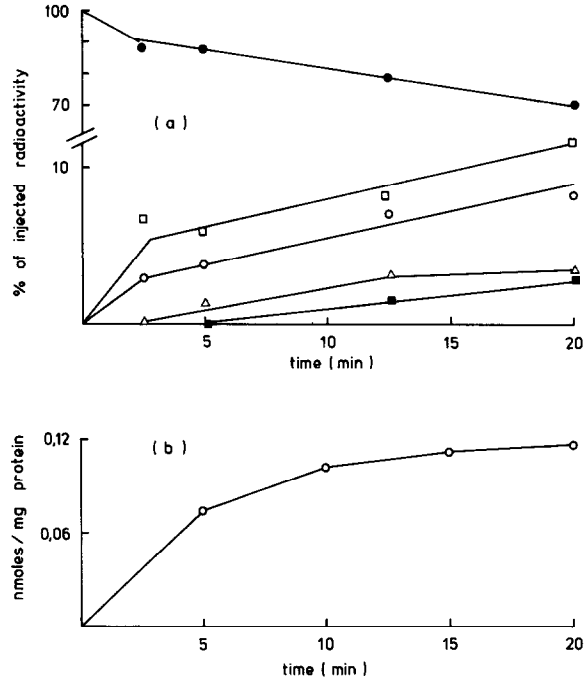

Fig.4. Fatty acid products of peroxisomal oxidation of [14-^{14}C]erucoyl-CoA. Peroxisomes (1.5 mg protein) were incubated as in section 2, the total incubation volume being 3.5 ml. Samples (0.6 ml) were withdrawn at the time intervals indicated in the figure, and mixed with 0.3 ml 5 M KOH. After 30 min at 85°C they were cooled, acidified with HCl, and extracted twice with 5 ml chloroform/methanol (2:1, v/v). After methylation the samples were analysed by radio-gas–liquid chromatography as in section 2. Samples (0.2 ml) from the same incubation were withdrawn at the same time-intervals for measurement of acid-soluble radioactivity. In (a) $C_{14:1}$, $C_{16:1}$, $C_{18:1}$, $C_{20:1}$, $C_{22:1}$ are indicated by (■), (△), (○), (□) and (•), respectively. The rate of disappearance of $C_{22:1}$ (1.01) after ~3 min incubation, is close to the sum of rates of appearance of $C_{16:1}$ (0.31), $C_{18:1}$ (0.34) and $C_{20:1}$ (0.38). In (b) acid-soluble radioactivity data have been expressed in terms of nmol [14-^{14}C]erucoyl-CoA oxidised to give acid-soluble radioactivity per mg peroxisomal protein.

Because measurable quantities of acid-soluble radioactivity were formed by peroxisomal oxidation of [14-^{14}C]erucoyl-CoA, a fraction of the substrate must be oxidised beyond the five cycles of oxidation required to generate acid-soluble radioactivity (~0.3% of the added [14-^{14}C]erucoyl-CoA, see fig.4). The failure to detect this by radio-gas–liquid chromatography is probably due to the poorer sensitivity of the proportional counter relative to the liquid scintillation spectrometer. It is also noteworthy that the intermediates which accumulate are identical to those found when erucic acid chain shortening was stimulated by NADP [7].

The results presented here, in our view, support the proposal [17] that peroxisomal β-oxidation may be of significance as regards the cellular oxidation of long chain mono-unsaturated fatty acids, at least as far as clofibrate-treated animals are concerned. The products of peroxisomal oxidation should be well suited for subsequent oxidation by mitochondrial β-oxidation.

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