Peroxisomal $\beta$-oxidation of long-chain fatty acids possessing different extents of unsaturation

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Rates of peroxisomal $\beta$-oxidation were measured as fatty acyl-CoA-dependent NAD$^+$ reduction, by using solubilized peroxisomal fractions isolated from livers of rats treated with clofibrate. Medium- to long-chain saturated fatty acyl-CoA esters as well as long-chain polyunsaturated fatty acyl-CoA esters were used. Peroxisomal $\beta$-oxidation shows optimal specificity towards long-chain polyunsaturated acyl-CoA esters. Eicosa-8,11,14-trienoyl-CoA, eicosa-11,14,17-trienoyl-CoA and docosa-7,10,13,16-tetraenoyl-CoA all gave $V_{\text{max}}$ values of about 150% of that obtained with palmitoyl-CoA. The $K_m$ values obtained with these fatty acyl-CoA esters were $17 \pm 6$, $13 \pm 4$ and $22 \pm 3 \mu M$ respectively, which are in the same range as the value for palmitoyl-CoA (13.8 $\pm$ 1 $\mu M$). Myristoyl-CoA gave the higher $V_{\text{max}}$ (110% of the palmitoyl-CoA value) of the saturated fatty acyl-CoAs tested. Substrate inhibition was mostly observed with acyl-CoA esters giving $V_{\text{max}}$ values higher than 50% of that given by palmitoyl-CoA.

INTRODUCTION

Several reports suggest that peroxisomal $\beta$-oxidation is most active towards medium- to long-chain acyl-CoA esters (Osumi & Hashimoto, 1978; Hryb & Hogg, 1979; Alexson & Cannon, 1984), and that it is more active towards long-chain monounsaturated fatty acids as compared with the corresponding saturated fatty acids (Osmundsen et al., 1979; Neat et al., 1981; Alexson & Cannon, 1984). Peroxisomal $\beta$-oxidation is considered to be physiologically relevant as regards chain shortening of fatty acids which are poorly oxidized by mitochondrial $\beta$-oxidation (for review see Osmundsen et al., 1987). We have previously obtained evidence suggesting that rat liver peroxisomal $\beta$-oxidation has appreciable ability to chain-shorten polyunsaturated fatty acids, e.g. linolenic acid or arachidonic acid (Hiltunen et al., 1986). Also fatty acids which are relatively slowly oxidized by mitochondrial $\beta$-oxidation, e.g. docosahexaenoic acid and $\gamma$-linolenic acid (Osmundsen & Bjørnstad, 1985), can be shown to be oxidized relatively rapidly by peroxisomal $\beta$-oxidation (Hiltunen et al., 1986). We have also observed that linoleic acid and linolenic acid are oxidized by peroxisomal $\beta$-oxidation appreciably faster than, e.g., oleic acid (Hiltunen et al., 1986). It is therefore possible that peroxisomal $\beta$-oxidation also may have a function as regards catabolism of polyunsaturated fatty acids and their metabolites.

We have carried out a more systematic investigation of peroxisomal rates of $\beta$-oxidation of long-chain fatty acids with chain lengths of 20 and 22 carbon atoms, and possessing from zero to six double bonds. The results of this investigation clearly show that, among these fatty acids, peroxisomal $\beta$-oxidation shows a preference for the more polyunsaturated ones. Fatty acids possessing a $\Delta^6$ double bond, however, appear to be an exception from this rule, the examples being arachidonic acid and eicosapentaenoic acid.

EXPERIMENTAL

Materials

Palmitoyl-CoA, CoA (grade 1-L), NAD$^+$ (grade III), eicosapentaenoic acid and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Lauric, myristic, stearic, eicosanoic, docosanoic, eicosa-11-enoic, eicosa-11,14-dienoic, eicosa-8,11,14-trienoic (homo-$\gamma$-linolenic acid), eicosa-11,14,17-trienoic, docosa-11-enoic, docosa-13,16-dienoic, docosa-13,16,19-trienoic, docosa-7,10,13,16-tetraenoic and docosa-4,7,10,13,16,19-hexaenoic acids were purchased from Nu-Chek Prep, Elysian, MN, U.S.A. Clofibrate was obtained from Fluka A.G., Buchs, Switzerland.

Synthesis of acyl-CoA esters

Acyl-CoA esters were synthesized essentially as described by Kawaguchi et al. (1980). Concentrations of acyl-CoA esters were measured enzymatically by using partially purified carnitine palmitoyltransferase (EC 2.3.1.21) as described by Osmundsen et al. (1979). Synthesis of unsaturated acyl-CoA esters was always carried out in solvents containing 0.005% (w/v) butylated hydroxytoluene as antioxidant. All unsaturated acyl-CoA esters were freshly prepared before use.

Isolation of peroxisomal fractions

Liver peroxisomal fractions were prepared from male albino Wistar rats which had been given fodder containing clofibrate (0.5%, w/w) for 2 weeks. Peroxisomal fractions were prepared in self-generated Percoll density gradients, with centrifugation in the VTi 50 rotor as described by Neat et al. (1981).

Protein assay

Protein was assayed with the Bio-Rad protein assay kit, with freeze-dried $\gamma$-globulin as standard.
Measurement of peroxisomal β-oxidation

This was done spectrophotometrically as acyl-CoA-dependent NAD+ reduction. The assay medium contained 1.0 mm-dithiothreitol, 0.50 mm-NAD+, 0.20 mm-CoA, 20 μM-FAD, 0.005% (v/v) Triton X-100 and 30 mm-potassium phosphate buffer, pH 7.50. The concentration of acyl-CoA was varied from about 2 to 120 μM. The total assay volume was 1 ml. All assays were carried out at 30 °C. Rates of NADH generation were measured at 340 nm with an Uvikon 810 spectrophotometer.

Regression analysis

The resulting data-sets of rates against substrate concentrations were analysed by non-linear regression analysis by using the PENNZYME program (Kohn et al., 1979; Schremmer et al., 1984), kindly supplied by Dr. J. Garfinkel, Moore School of Electrical Engineering, Philadelphia, PA, U.S.A. The data were fitted to a conventional Michaelis–Menten model, incorporating a term to accommodate the apparent substrate inhibition (see Palmer, 1985), as substrate inhibition was apparent with many of the substrates. The computed parameters were \( V_{\text{max}}, K_m \) and the substrate-inhibition constant \( K_i \). Sets of data showing no evidence of substrate inhibition were fitted to the standard Michaelis–Menten equation. All computations were carried out on an IBM XT personal computer. \( V_{\text{max}} \) values are expressed as percentage of that obtained with palmitoyl-CoA (135 ± 15 nmol of NADH produced/min per mg of protein; \( n = 3 \)). Starting estimates of parameter values were obtained graphically from Lineweaver–Burk plots. The regression analysis was a two-stage process: an initial analysis using a Simplex procedure, followed by Fletcher–Powell analysis using the Simplex program parameter estimates as starting values (Kohn et al., 1979; Schremmer et al., 1984). All data sets used (shown in Figs. 1 and 2) were found to converge within the interaction limit (10 times the number of parameters, i.e. 30 interactions for a model incorporating substrate inhibition). The resulting parameters were all statistically significant with respect to the model used.

RESULTS AND DISCUSSION

All fatty acyl-CoA esters tested were found to be substrates for peroxisomal β-oxidation, although they were β-oxidized at markedly different rates. Most of the fatty acyl-CoA esters showed substrate inhibition; the inhibition constants \( (K_i) \) obtained are given in Table 1. Plots of rates versus substrate concentration for the C\( 20:0 \) and C\( 22:0 \) series of acyl-CoA esters are shown in Figs. 1 and 2 respectively. From these results it is immediately apparent that rates of peroxisomal β-oxidation increase with increasing extent of unsaturation of the carbon chain. This is in agreement with results obtained with the C\( 18:1 \) series, showing γ-linolenoyl-CoA being a better substrate than oleoyl-CoA and linoleoyl-CoA (Os mundsen et al., 1987), and linolenic acid being a better substrate than oleic acid and linoleic acid (Hiltunen et al., 1986). However, eicosapentaenooyl-CoA (C\( 20:5 \)-CoA) and docosahexaenooyl-CoA (C\( 22:6 \)-CoA) are β-oxidized at much lower rates.

The kinetic parameters \( V_{\text{max}} \) and \( K_m \) found with the
various fatty acyl-CoA esters are shown in Figs. 3 and 4. Within the $C_{20:n}$ series the $V_{\text{max}}$ value increases with increasing degree of unsaturation. The $K_m$ values are high for the saturated substrates and low for the unsaturated ones, except for eicosapentaenoyl-CoA ($C_{20:5}$-CoA) (Fig. 3a). Homo-$\gamma$-linolenoyl-CoA (eicosa-8,11,14-trienoyl-CoA) gave a $K_m$ value of 17 ± 6 $\mu$m and a $V_{\text{max}}$ value of 170 ± 40%, not significantly different from those obtained with eicosa-11,14,17-trienoyl-CoA. The similarity of the kinetic parameters obtained with the two $C_{22:n}$ isomers suggest that the positioning of the additional double bond in either the $\Delta^2$ or the $\Delta^{17}$ position is kinetically equivalent.

For the $C_{22:n}$ series rates of $\beta$-oxidation increased with increasing degree of unsaturation, in a manner similar to that observed with the $C_{20:n}$ series. The exception was docosahexaenoyl-CoA ($C_{22:6}$-CoA) (Fig. 3b). However, docosadienoyl-CoA ($C_{22:2}$-CoA) has a higher $V_{\text{max}}$ than docosatrienoyl-CoA ($C_{22:3}$-CoA), but the $V_{\text{max}}/K_m$ ratio is lower. Therefore at low substrate/enzyme ratios docosatrienoyl-CoA is the better substrate.

Arachidonoyl-CoA ($C_{20:4}$-CoA) has previously been shown to be relatively slowly $\beta$-oxidized (Hiltunen et al., 1986; Osmundsen et al., 1987). Results presented here show that also eicosapentaenoyl-CoA ($C_{20:5}$-CoA) is slowly $\beta$-oxidized. Why both arachidonoyl-CoA and eicosapentaenoyl-CoA are relatively poor substrates is not clear. Both of these fatty acids, however, possess a
The $K_m$ values are low and relatively constant for lauroyl-CoA ($C_{12:0}$-CoA) to stearoyl-CoA ($C_{18:0}$-CoA), and increase markedly for eicosanoyl-CoA ($C_{20:0}$-CoA) and docosanoyl-CoA ($C_{22:0}$-CoA) (see Fig. 4). This is in agreement with results obtained previously for saturated fatty acyl-CoA esters (Osumi & Hashimoto, 1978; Hryb & Hogg, 1979; Neat et al., 1981; Alexson & Cannon, 1984). The calculated absolute maximal velocity found now with palmitoyl-CoA (135 ± 15 nmol of NADH/min per mg of protein) is about half that reported by Lazarow (1978). This difference may be due to different extents of induction of peroxisomal β-oxidation, as well as a difference in purity of the peroxisomal fractions used.

Among the fatty acyl-CoA esters tested, some long-chain polysaturated acyl-CoA esters are found to be better substrates. Earlier reports often claimed medium-chain saturated fatty acids or long-chain monounsaturated fatty acids to be the better substrates for peroxisomal β-oxidation, but those did not include polysaturated fatty acids (Osumi & Hashimoto, 1978; Hryb & Hogg, 1979; Alexson & Cannon, 1984).

The substrates giving highest $V_{max}$ values, i.e. eicos-8,11,14-trienoyl-CoA (homoy-linolenoyl-CoA), eicos-11,14,17-trienoyl-CoA ($C_{20:3}$-CoA) and docosa-7,10,13,16-tetraenoyl-CoA ($C_{22:4}$-CoA), all have high $V_{max}$ values, around 150%, and low $K_m$ values, 13–22 μM. These fatty acyl-CoA esters gave higher activity than palmitoyl-CoA at all substrate concentrations below those where substrate inhibition greatly influences the observed rate. These faster rates are due to higher $V_{max}$ values, since the $K_m$ values are similar.

The $K_m$ values for the substrates giving high or intermediate rates of reaction range from 10 to 20 μM, whereas those of the poorer substrates are about 40–50 μM. This represents an increase of about 4-fold, but the $K_m$ values are still rather low. The $V_{max}$ values, on the other hand, are around 150% for the better substrates, and they gradually decrease with substrates giving lower rates of oxidation, down to a value of 40% for eicosanoyl-CoA, and only 3.9% for docosanoyl-CoA. The discrimination between the substrates is thus more of a $V_{max}$ effect than a $K_m$ effect.

Acyl-CoA oxidase is most probably the rate-limiting enzyme of peroxisomal β-oxidation (Osumi & Hashimoto, 1979; Inestrosa et al., 1979). The $K_m$ values of this enzyme with some saturated fatty acyl-CoA esters (lauroyl-, myristoyl-, palmitoyl- and stearoyl-CoA) (Osumi et al., 1980) are practically the same as those observed here for peroxisomal β-oxidation. The variations in the $V_{max}$ values are very similar too, the exception being the lower $V_{max}$ value for myristoyl-CoA, compared with palmitoyl-CoA (Osumi et al., 1980). This, together with the similarity of pattern of substrate inhibition, is therefore in agreement with acyl-CoA oxidase being the rate-limiting enzyme in peroxisomal β-oxidation.

Most fatty acyl-CoAs used showed substrate inhibition. In general the better substrates have the lowest $K_i$ values, i.e. they are the more potent inhibitors. The mechanism of substrate inhibition is difficult to elucidate, as rates of acyl-CoA-dependent NADH-reduction have been measured. This involves a set of reactions, where the first reaction is likely to be rate-limiting. The similarity between our results and those of Osumi et al. (1980) suggests that the observed substrate inhibition may be

![Fig. 4. Effect of carbon-chain length on kinetic parameters obtained with saturated acyl-CoA esters](image-url)
Specificity of peroxisomal $\beta$-oxidation

due to non-productive binding of substrate to acyl-CoA oxidase.

Chain shortening of long-chain fatty acids, which are poorly oxidized by mitochondria, has been considered the main physiological role of peroxisomal $\beta$-oxidation (Osmundsen et al., 1987). The high activity observed here with long-chain polyunsaturated acyl-CoA esters suggests that other of their metabolites, e.g. prostanoids, may also be catabolized by peroxisomal $\beta$-oxidation.

We thank Mr. T. Braum for excellent technical assistance. This investigation was supported by The Norwegian Council for Science and Humanetics, and Nordisk Insulin Fond.

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Received 3 April 1987/1 June 1987; accepted 21 July 1987

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