Intermediates of peroxisomal β -oxidation

A study of the fatty acyl-CoA esters which accumulate during peroxisomal β -oxidation of [U- 14 C]hexadecanoate

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1. 14 C-labelled fatty acyl-CoA esters resulting from β -oxidation of [U- 14 C]hexadecanoate by peroxisomal fractions isolated from rats treated with clofibrate showed the presence of the full range of saturated intermediates down to acetyl-CoA. 2. The pattern of intermediates generated was fairly constant. At low concentrations of [U- 14 C]hexadecanoate (50 μ M), decanoyl-CoA was present in lowest amounts. At higher concentrations of [U- 14 C]hexadecanoate (> 100 μ M), all intermediates of chain length shorter than 12 carbon atoms (except acetyl-CoA) were present at similar low concentrations; the process of β -oxidation now resembling chain-shortening of hexadecanoate by two cycles of β -oxidation. 3. In the absence of an NAD $^{+}$ -regenerating system [pyruvate and lactate dehydrogenase (EC 1.1.1.28)] 2-enoyl- and 3-hydroxyacyl-CoA esters were generated, suggesting that re-oxidation of NADH is essential for optimal rates of peroxisomal β -oxidation *in vitro*. 4. At high concentrations of [U- 14 C]hexadecanoate (> 100 μ M), 3-oxohexadecanoyl-CoA was produced, suggesting that thiolase (acetyl-CoA acetyltransferase; EC 2.3.1.9) can become rate-limiting for peroxisomal β -oxidation.

INTRODUCTION

Peroxisomal β -oxidation of long-chain fatty acids is known to be incomplete (for review, see Bremer & Osmundsen, 1984). The precise extent of chain shortening remains to be unequivocally established, although the occurrence of three to five cycles of β -oxidation has been reported for hexadecanoate (Osmundsen *et al.*, 1979; Lazarow, 1982). It has also been reported that a fraction of the available hexadecanoate molecules may be shortened as far as butyrate (Osmundsen, 1982). Results obtained with longer (unsaturated) fatty acids suggest that two or three cycles of β -oxidation occur (Hiltunen *et al.*, 1986; Osmundsen & Hovik, 1988). Measurement of chain-shortening of [14 C]erucic acid *in vivo* also suggested that two cycles of β -oxidation is the predominant situation with respect to this substrate (Christiansen *et al.*, 1979).

Using isolated peroxisomal fractions we have examined the acyl-CoA intermediates resulting from β -oxidation of [U- 14 C]-hexadecanoic acid. We have examined the effects of fatty acid and CoA concentration on the pattern of intermediates, as well as the effect of an added NAD $^{+}$ -regenerating system, i.e. pyruvate plus lactate dehydrogenase (EC 1.1.1.28). Our data show that all saturated acyl-CoA esters down to acetyl-CoA are generated, although often in significantly different amounts. Depending on the conditions of incubation, 2-enoyl-, 3-hydroxyl- and 3-oxo-acyl-CoA esters can also be detected.

EXPERIMENTAL

Materials

CoA, acetyl-CoA, n-heptanoyl-CoA, n-pentadecanoyl-CoA, FAD, NAD $^{+}$, NADP $^{+}$, Triton X-100, antimycin a, BSA (essentially fatty-acid free) and potassium pyruvate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All fatty acids were purchased from Fluka, Buchs, Switzerland. Percoll was obtained from Pharmacia, Uppsala, Sweden. [U- 14 C]Hexadecanoic acid was purchased from Amersham International.

Animals

Male Wistar albino rats were purchased from Veterinær Møllegaards Avlsstasjon, Havdrup, Denmark. The rats had body weights of about 200 g at the time of experimental use. The rats were treated with clofibrate [0.5 % (w/w) in the food] for about 10 days before use.

Isolation of rat liver peroxisomal fractions

Rat liver peroxisomal fractions were isolated in a self-generated Percoll gradient as described previously (Neat *et al.*, 1981; Osmundsen, 1982). The isolated fractions were used in incubations immediately after isolation.

Peroxisomal incubations and assay of peroxisomal β -oxidation

Isolated peroxisomal fractions were incubated using non-solubilizing (iso-osmotic) incubation conditions. The incubations contained (unless otherwise stated) 130 mM-KCl, 20 mM-Hepes, 0.1 mM-EGTA, 0.5 mM-NAD $^{+}$, 0.1 mM-NADP $^{+}$, 0.2 mM-CoA, 10 mM-MgATP, 0.1 mM-dithiothreitol, 2 units of lactate dehydrogenase/ml, 20 mM-pyruvate, 10 μ g of antimycin a/ml and 2 mg of defatted BSA/ml, pH 7.2. The substrate was always [U- 14 C]hexadecanoate (specific radioactivity 10000 d.p.m./nmol), which was added as a complex with BSA. Unless otherwise indicated, substrate concentration was 50 μ M. The incubations contained about 0.5 mg of peroxisomal protein/ml. All incubations were carried out at 37 °C in a shaking water bath.

Peroxisomal β -oxidation of [U- 14 C]hexadecanoate was measured as acid-soluble radioactivity released after various periods of incubation. Usually, samples (100 μ l) of the incubation mixtures were withdrawn and added to an equivalent volume of ice-cold HClO $_4$ (10 %, v/v). Following a brief centrifugation to remove denatured proteins, the radioactivity in the supernatants was measured.

The peroxisomal fractions contain a minor mitochondrial contaminant (see Neat *et al.*, 1981). With these conditions of incubation, however, the rate of β -oxidation of labelled hexadecanoyl carnitine has been shown to be less than 10 % of that of

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labelled hexadecanoyl-CoA (Osmundsen, 1982). When free hexadecanoate is used as a peroxisomal substrate, the resulting hexadecanoyl-CoA is essentially unavailable for mitochondrial β -oxidation because no carnitine has been added to the incubations. It is also well established that few, if any, intermediates accumulate during mitochondrial β -oxidation (Watmough *et al.*, 1988, 1989). It is therefore most unlikely that a significant portion of the intermediates observed was due to contaminating mitochondrial activity.

Analysis of radioactive acyl-CoA esters by radio-h.p.l.c.

Incubations were quenched with glacial acetic acid and heptadecanoyl-CoA was added as internal standard. Acyl-CoA esters were extracted with methanol/chloroform (2:1, v/v) and prepared for h.p.l.c. analysis as previously described (Watmough *et al.*, 1989). Preliminary analysis showed that the DEAE-Sephacel fractionation was unnecessary, and this procedure was therefore omitted. Acyl-CoA esters were analysed by h.p.l.c. with on-line radiochemical and photodiode-array detection, as described previously (Watmough *et al.*, 1988, 1989), except that a 5 μ Hypersil C₁₈ column was used.

Assay of protein

Proteins were assayed using the Bio-Rad protein assay kit, with freeze-dried γ -globulin as standard.

RESULTS

Effects of pyruvate plus lactate dehydrogenase on peroxisomal β -oxidation

Fig. 1 shows typical time courses of β -oxidation of [U-¹⁴C]hexadecanoate

Fig. 1. Time course of peroxisomal β -oxidation of [U-¹⁴C]hexadecanoate in the presence and absence of added pyruvate and lactate dehydrogenase

Isolated peroxisomal fractions were incubated with 50 μ M-[U-¹⁴C]hexadecanoate as described in the Experimental section, in the absence (B) and the presence (A) of 10 mM-pyruvate plus 2 units of lactate dehydrogenase/ml. At the time points indicated, samples of the incubations (100 μ l) were removed for measurement of acid-soluble radioactivity, as described in Experimental section. The incubations contained 0.7 mg of peroxisomal protein/ml.

[¹⁴C]hexadecanoate obtained using standard conditions of incubation, and also when no pyruvate or lactate dehydrogenase had been added. The results show that peroxisomal β -oxidation was stimulated by about 50% by pyruvate plus lactate dehydrogenase (Fig. 1). Additional experiments (results not shown) have shown this stimulation to vary from 10 to 60% in different peroxisomal fractions. The reason for this is not clear. A similar stimulatory effect of a NAD⁺-regenerating system has been described previously using fatty acyl-CoA esters as substrates for peroxisomal β -oxidation (Osmundsen, 1982).

Samples taken at identical time intervals were also analysed by radio-h.p.l.c. for [¹⁴C]-labelled acyl-CoA esters (Fig. 2). Analysis of [¹⁴C]acyl-CoA esters by radio-h.p.l.c. showed that appreciable amounts of [U-¹⁴C]hexadecanoyl-CoA were present in the incubation mixture after 1 min of incubation, although a significant amount of chain-shortened acyl-CoA ester ([¹⁴C]tetradecanoyl-CoA) was seen only in incubations containing pyruvate plus lactate dehydrogenase (Fig. 2). After 2 min of incubation, [¹⁴C]acetyl-CoA was detectable in both incubations, but [¹⁴C]tetradecanoyl-CoA and [¹⁴C]dodecanoyl-CoA were only readily detectable in incubations containing pyruvate plus lactate dehydrogenase. With longer periods of incubation, all intermediate acyl-CoA esters, down to and including [¹⁴C]butyryl-CoA, were present under both set of incubation conditions.

Although the amount of intermediates were consistently markedly higher in incubations containing pyruvate plus lactate dehydrogenase, the patterns of intermediates were remarkably similar both in the presence and absence of the NAD⁺-regenerating system (Figs. 2a and 2b). In practically all samples [¹⁴C]decanoyl-CoA and [¹⁴C]octanoyl-CoA represented the intermediates present in the lowest amounts, with the amount of [¹⁴C]decanoyl-CoA usually being lowest (Figs. 2a and 2b).

In the presence of pyruvate plus lactate dehydrogenase, only saturated intermediates were observed, whereas in their absence small amounts of [¹⁴C]tetradec-2-enoyl-CoA, [¹⁴C]hexadec-2-enoyl-CoA and [¹⁴C]3-hydroxyhexadecanoyl-CoA were also found (Fig. 2). An additional peak of radioactivity was observed with a retention time of 29 min (Figs. 2b and 2d). This peak is probably a mixture of [¹⁴C]3-hydroxytetradecanoyl-CoA and [¹⁴C]dodec-2-enoyl-CoA. Their presence suggest that the pyruvate plus lactate dehydrogenase-mediated re-oxidation of NADH has removed a rate-limiting step for peroxisomal β -oxidation. These findings therefore confirm and extend the previously observed stimulatory effect of an NAD⁺-regenerating system on intermediates of peroxisomal β -oxidation (Osmundsen, 1982).

Effects of concentration of added CoA on intermediates of β -oxidation

We have previously demonstrated that non-esterified fatty acids are readily β -oxidized by isolated peroxisomal fractions provided that MgATP and CoA are added to the incubations (Hiltunen *et al.*, 1986; Osmundsen *et al.*, 1987). We have therefore investigated the effect of the exogenous CoA concentration on the rate of β -oxidation, correlating this with radio-h.p.l.c. analysis of [¹⁴C]acyl-CoA esters present in the incubations. The results show that β -oxidation of [U-¹⁴C]hexadecanoate is critically

Fig. 2. Radio chromatograms of [¹⁴C]acyl-CoA esters extracted from peroxisomal incubations

Isolated peroxisomal fractions were incubated with 50 μ M-[U-¹⁴C]hexadecanoate as described in the legend to Fig. 1. At the time intervals indicated, samples (300 μ l) were removed and added to 300 μ l of ice-cold glacial acetic acid. The acyl-CoA esters were subsequently extracted and chromatographed as described in the Experimental section. Peaks of radioactivity were integrated to quantify the amounts of the various acyl-CoA esters. The resulting data are presented as histograms, for incubations in the presence (a), and absence (b) of 10 mM-pyruvate plus 2 units of lactate dehydrogenase/ml. Also presented are typical radiochromatograms derived from samples removed after 2 and 20 min of incubation in the presence (c) or absence (d) of 10 mM-pyruvate plus 2 units of lactate dehydrogenase/ml. The identities of the various peaks of radioactivity are indicated: C₁₆, [¹⁴C]hexadecanoyl-CoA; C_{16:1}, [¹⁴C]hexadec-2-enoyl-CoA; C₁₄, [¹⁴C]tetradecanoyl-CoA; C_{14:1}, [¹⁴C]tetradec-2-enoyl-CoA; C₁₂, [¹⁴C]dodecanoyl-CoA; C₁₀, [¹⁴C]decanoyl-CoA; C₈, [¹⁴C]octanoyl-CoA; C₆, [¹⁴C]hexanoyl-CoA; C₄, [¹⁴C]butyryl-CoA; C₂, [¹⁴C]acetyl-CoA.

Fig. 2. a, b, c, d.

Fig. 3. Effects of various concentrations of CoA on peroxisomal β -oxidation and on acyl-CoA ester intermediates generated

Isolated peroxisomal fractions were incubated with 50 μM -[U- ^{14}C]hexadecanoate in the presence of various concentrations with CoA. The incubations contained 0.8 mg of peroxisomal protein/ml. After 20 min of incubation the samples were quenched for measurement of acid-soluble radioactivity (a) and for radio-h.p.l.c. analysis of [^{14}C]acyl-CoA esters as described in the Experimental section. The peaks of radioactivity were integrated and resulting data are presented as histograms (b).

dependent on the concentration of added CoA, at least up to 200 μM (Fig. 3a).

The amounts of CoA esters formed are, as expected, also extremely dependent on the concentration of added CoA. When CoA was included to a concentration of 25 μM , only small amounts of hexadecanoyl-CoA and tetradecanoyl-CoA were present in the incubations. Further increases in the concentration of added CoA led to the appearance of increasing amounts of a full complement of intermediates. As shown in Fig. 3(b), [^{14}C]decanoyl-CoA is invariably the least abundant intermediate. In addition, at lower concentrations of CoA, a peak which co-chromatographs with 3-oxohexadecanoyl-CoA is observed.

Effects of the concentration of [U- ^{14}C]hexadecanoate on the pattern of acyl-CoA intermediates

Fig. 4 shows the patterns of [^{14}C]acyl-CoA intermediates obtained using concentrations of [U- ^{14}C]hexadecanoate ranging

from 25 to 400 μM . These results can be correlated with rates of β -oxidation, measured as acid-soluble radioactivity released at the various concentrations of [U- ^{14}C]hexadecanoate used. The concentration of added CoA was kept at 200 μM , and all samples were incubated for 20 min. Again, [^{14}C]decanoyl-CoA and [^{14}C]octanoyl-CoA represented the two least abundant intermediates. With increasing concentrations of [U- ^{14}C]hexadecanoate, however, this pattern becomes increasingly less prominent. At a concentration of 400 μM -[U- ^{14}C]hexadecanoate, all intermediates between dodecanoyl-CoA and acetyl-CoA were present at about the same level. These chain-shortened intermediates. With increasing concentrations of [U- ^{14}C]hexadecanoate, however, this pattern becomes increasingly less pro-

Fig 4(b) also shows that the amount of [U- ^{14}C]3-oxohexadecanoyl-CoA was increased several-fold as the concentration of [U- ^{14}C]hexadecanoate was increased to 200 μM , suggesting that thiolase (acetyl-CoA acetyltransferase; EC 2.3.1.9) activity may

Fig. 4. Effects of various concentrations of [U- ^{14}C]hexadecanoate on peroxisomal β -oxidation and on acyl-CoA ester intermediates generated

Isolated peroxisomal fractions were incubated as described in the Experimental section with various concentrations of hexadecanoate. The incubations contained 0.6 mg of peroxisomal protein/ml. After 20 min of incubation the samples were quenched for measurement of acid-soluble radioactivity (a) and for radio-h.p.l.c. analysis of [^{14}C]acyl-CoA esters as described in the Experimental section. The peaks of radioactivity were integrated and resulting data are presented as histograms (b).

become rate-limiting for β -oxidation at high fatty acid concentrations. At a concentration of [U- ^{14}C]hexadecanoate of 400 μM the rate of β -oxidation was decreased, even though the amount of [U- ^{14}C]hexadecanoyl-CoA which had been formed was increased. The reason for this is not clear, but may be related to substrate inhibition of the acyl-CoA oxidase by hexadecanoyl-CoA (Hovik & Osmundsen, 1987).

DISCUSSION

The reason for partial β -oxidation by peroxisomes has never been explained. The data presented here shed some light on the mechanism. Our results demonstrate that peroxisomal β -oxidation is not limited to a few cycles. This is particularly apparent when a low concentration of [U- ^{14}C]hexadecanoate is used (Fig. 4). At concentrations of 25 or 50 μM [U- ^{14}C]hexadecanoate, intermediates of chain length shorter than 12 carbon atoms (excluding acetyl-CoA) constituted about 60% and 50% res-

pectively of the total amount of chain-shortened [^{14}C]acyl-CoA esters. At higher concentrations of [U- ^{14}C]hexadecanoate, however, these intermediates only constituted about 20% of the total. At these higher concentrations of fatty acid, therefore, the characteristics of peroxisomal β -oxidation approach simple chain-shortening of the substrate by up to two cycles of β -oxidation. Hence it appears that when peroxisomes are exposed to a high concentration of fatty acid, chain-shortening similar to that inferred from studies *in vivo* (Christiansen, 1978) ensues.

These observations also explain previous discrepancies regarding the extent of chain-shortening reported with respect to hexadecanoyl-CoA (Lazarow & de Duve, 1976; Osmundsen *et al.*, 1979; Osmundsen, 1982). The extent of chain-shortening observed is likely to have varied with concentration of substrate, conditions of incubation and the sensitivity of the technique used to monitor chain-shortening.

The patterns of intermediates observed using a concentration of [U- ^{14}C]hexadecanoate of 50 μM are remarkably similar, ir-

respective of conditions of incubation. Decanoyl-CoA is invariably the intermediate present at the lowest concentration (Figs. 2, 3 and 4). The concentration of longer-chain-length saturated intermediates always increased with increasing chain length. With shorter intermediates the concentration also increased, with chain length decreasing to 6-carbon atoms (hexanoyl-CoA). The concentration of butyryl-CoA, however, was always markedly lower than that of hexanoyl-CoA. This pattern was greatly dependent on the concentration of [U-¹⁴C]hexadecanoate (Fig. 4), as discussed previously. The reason for this pattern of intermediates remains unclear. However, Alexson *et al.* (1989) have shown that the acyl-CoA hydrolase activity in brown adipose tissue peroxisomes increased markedly with decreasing chain length of the fatty acyl-CoA substrate. Maximal activity was observed with substrates of chain length of between three and twelve carbon atoms. It is therefore plausible that acyl-CoA hydrolases may have a function in the termination of chain-shortening, assuming the existence of a similar pattern of activity in liver peroxisomes. The observed pattern of acyl-CoA intermediates reported here may therefore represent the balance between the flux through successive cycles of β -oxidation and the activity of the acyl-CoA hydrolase(s) towards the acyl-CoA esters which are generated.

It has recently been suggested that acetyl-CoA derived from peroxisomal β -oxidation in isolated hepatocytes can emerge as acetate (Leighton *et al.*, 1989). In our time courses of β -oxidation intermediate production, the level of acetyl-CoA did not increase linearly with time, but rather increased towards a limiting value and subsequently declined (Figs. 2a and 2b). The reason for this is not entirely clear, although an acetyl-CoA hydrolase activity (with an apparent K_m of about 0.4 mM for acetyl-CoA) can be detected in purified peroxisomal fractions (R. Hovik &

H. Osmundsen, unpublished work). The observed variations in the concentration of acetyl-CoA could be due to this activity.

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