Production of formyl-CoA during peroxisomal α-oxidation of 3-methyl-branched fatty acids

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Abstract α-Oxidation of 3-methyl-substituted fatty acids was studied in purified rat liver peroxisomes. The experiments revealed that formyl-CoA is formed during the α-oxidation process. The amount of formyl-CoA found constituted 2–5% of the amount of formate formed. Under the conditions used, no activation of exogenously added formate occurred in purified peroxisomes, whereas 95.5% of added synthetic formyl-CoA was converted to formate. These data indicate that during α-oxidation first formyl-CoA is formed, which is then hydrolysed to formate.

Key words: Hydrolase; Phytanic acid; Refsum's disease

1. Introduction

3-Methyl-substituted fatty acids cannot undergo peroxisomal β-oxidation because of the presence of a methyl group in the 3-position. They first have to be shortened by one carbon atom via α-oxidation resulting in 2-methyl-substituted fatty acids [1], that are substrates for peroxisomal β-oxidation [2–6]. Degradation of phytanic acid, a 3-methyl-branched fatty acid [1], and of synthetic 3-methyl-branched fatty acids [4] is deficient in Refsum's disease and in disorders of peroxisome biogenesis, which leads in these disorders to progressive accumulation of phytanic acid. Mihalik et al. [7] and Croes et al. [8] have recently demonstrated that in rat liver α-oxidation is a peroxisomal process. Casteels, Croes, Van Veldhoven and Mannaerts (submitted) have demonstrated this for human liver as well, which classifies Refsum's disease as a peroxisomal disorder. Mihalik et al. [7] and Croes et al. [8] have also shown that the first step of α-oxidation is an activation reaction, which is followed by a 2-hydroxylation [8] and they were able to identify a 2-hydroxy-3-methylacyl-CoA intermediate [7,8]. As shown first by Poulos et al. in human fibroblasts [9] and later by Casteels et al. [10] in isolated rat hepatocytes, besides CO_2 also formate is produced by α-oxidation. It has recently been demonstrated by Croes et al. [8] that formate is in fact the primary end-product that is rapidly converted to CO_2. The reactions starting from the 2-hydroxy-3-methylacyl-CoA intermediate and leading to the production of formate remain largely unknown.

Considering the fact that a 2-hydroxy-3-methylacyl-CoA intermediate and formate are formed, one may expect formyl-CoA to be generated in an intermediary step. As far as we know, however, the existence of formyl-CoA has up to now only been described in microorganisms [11–13].

We, therefore, investigated whether formyl-CoA is formed during α-oxidation in purified peroxisomes, using the synthetic 3-methyl[1-^{14}C]hexadecanoic acid (3-methyl[1-^{14}C]-palmitic acid) as a substrate [4,14].

2. Materials and methods

2.1. Materials

Sodium [^{14}C]formate was from New England Nuclear. Unlabelled and 1-^{14}C-labelled (specific radioactivity 58.5 Ci/mol) 3-methylpalmitic acid were synthesized as described before [8]. Vivaspin 15 ml concentrators (cut off 10000 Da) were obtained from Sipan (Lier, Belgium).

2.2. Synthesis of formyl-CoA

For the synthesis of unlabelled and ^{14}C-labelled formyl-CoA, the reaction scheme of Rajgarhia et al. [15] for activation of acetate and propionate was followed, but the reaction volumes were doubled and the concentration of dichlorobenzoic acid was increased as this was required for a good solubilization of the formic acid.

Briefly, 20 μmol unlabelled or ^{14}C-labelled formate, sodium salt (0.23 Ci/mol) was dried in a 25 ml glass round-bottom flask over P_2O_5 for 48 h. Dichlorobenzoic acid (80 μmol) in dry tetrahydrofuran (1 ml) was added and the flask was sealed with a stopper and parafilm. The mixture was stirred with a magnetic stirrer at 65°C for 90 min. The reaction mixture was cooled to room temperature before the addition of 1,1′-carbonyldiimidazole (40 μmol) in dry tetrahydrofuran (1 ml) and was stirred at 65°C for another 90 min. The reaction mixture was cooled to room temperature. CoA (25 μmol) in aqueous imidazole buffer (1.6 ml, 0.5 M, pH 7.0) was added and the reaction mixture stirred at room temperature for 15 min. After acidification (pH 3), the solvent was evaporated under N_2 on ice. [^{14}C]Formic acid, likely to escape during evaporation, was trapped in 1 N NaOH. During the further purification the solution containing formyl-CoA was kept on ice or at 4°C at all times except when mentioned otherwise. The formyl-CoA was purified by isocratic elution monitored by radioactivity and/or UV-absorbance at 254 nm from an Econosphere C_{18} preparative column (Alltech, 250×22 mm, 10 μm, 80 Å) with acetonitrile/potassium phosphate buffer (50 mM, pH 5.5), 5/95 (v/v), at room temperature (flow rate was 5.0 ml/min). The formyl-CoA fractions were collected, acidified to pH 3 and pooled. To obtain a concentrated formyl-CoA solution in a low ionic strength medium, the acetonitrile (rotovap, 25–30°C) was removed and the salt solution containing formyl-CoA was applied at 4°C on an activated C_{18} SPE-column (Varian, 500 mg), equilibrated with 15 ml of potassium phosphate buffer (50 mM, pH 3). Formyl-CoA was eluted with 2 ml of potassium phosphate buffer (10 mM, pH 3)/acetonitrile (1:9, v/v) and 2 ml of methanol. The formyl-CoA fractions were collected and evaporated to dryness under N_2 on ice. The residue was reconstituted in 400 μl of distilled water in order to obtain a solution of formyl-CoA in potassium phosphate buffer (5 mM, pH 3) and stored at −20°C until use. Spectral analysis of the formyl-CoA, performed directly after purification, was in accordance with its structure (absorbance maximum at 260 nm (adenine group); ratio $A_{233}/A_{260} = 0.54$ (thioester)).

2.3. Animals

The experiments were performed with overnight-fasted male Wistar rats weighing 200–250 g.

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2.4. Preparation and incubation of purified peroxisomes

A light mitochondrial fraction of rat liver was prepared in 0.25 M sucrose containing 0.1% (v/v) ethanol and 5 mM 3-(*N*-morpholino)-propanesulfonic acid (pH 7.2) [16] and subfractionated by centrifugation through an iso-osmotic self-generating Percoll gradient as described before [8]. The gradient was collected in 30 fractions of equal volume starting from the bottom and the fractions containing the peroxisomes were pooled and diluted approximately 10-fold with the sucrose medium. After centrifugation for 20 min at 16200×*g* in a conical tube, the supernatant was removed and the peroxisomes were resuspended in 3 ml of sucrose medium. Incubations (37°C) were started by adding 100 μl of the purified peroxisomes (0.5–1.1 mg protein) to 400 μl of reaction medium. Final concentrations were 100 mM KCl, 50 mM Tris (pH 7.5), 0.025 mM defatted bovine serum albumin, 4 mM ATP, 2.4 mM $MgCl_2$, 0.2 mM CoA, 0.1 mM $FeCl_2$, 3 mM 2-oxoglutarate and 10 mM L-ascorbate. Substrate concentrations were 0.05 mM 3-methyl[1-^{14}C]palmitate (specific radioactivity 58.5 Ci/mol), 0.025 mM [^{14}C]formate (specific radioactivity 5 Ci/mol) and 0.2 mM [^{14}C]formyl-CoA (specific radioactivity 0.23 Ci/mol). Reactions were terminated after 10 min by acidification to pH 3 by addition of 50 μl of potassium phosphate (1 M, pH 2) and the reaction vials were immediately placed in ice water.

2.5. Analysis of the reaction medium for [^{14}C]formyl-CoA

After termination of the reaction, the reaction mixtures of duplicate samples were collected in an Eppendorf tube and centrifuged at 12000×*g* for 10 min at 4°C. The supernatant was filtered through a Vivaspin concentrator (cut off 10000 Da) by centrifugation at 1000×*g* for 90 min at 4°C. Filtrates were kept at −20°C until HPLC analysis the following day.

Unlabelled formyl-CoA (50 nmol in 7 μl) was added as a carrier to 750 μl of the filtrate (450 μl for 3-methyl[1-^{14}C]palmitate as a substrate), 700 μl (400 μl) of which was injected onto a Nova-Pak C_{18} HPLC column (3.9×150 mm, 4 μm, 60 Å).

Formate, CoA and formyl-CoA were separated with a gradient of acetonitrile in potassium phosphate buffer (50 mM, pH 5.5): 100% phosphate buffer for 3 min; linear increase 0–2% acetonitrile in 1 min; isocratic 2% acetonitrile in phosphate buffer for 26 min. Flow rate was 1.0 ml/min.

Column effluents were monitored by using an on-line UV-detector set at 254 nm. Radioactivity was monitored after the UV-detector by using a radioactivity detector connected in series (for incubations with 3-methyl[1-^{14}C]palmitate) as described before [8], or (for the other substrates with lower specific radioactivity) by collection of the fractions and liquid scintillation counting. Counting efficiency of the on-line detector was approximately 50%.

2.6. Determination of [^{14}C]formate on column effluents

Purified peroxisomes were incubated for 10 min with 3-methyl-[1-^{14}C]palmitate and incubation mixtures analysed as described above.

Column effluents were collected in fractions of 1 ml after passing the UV-detector. Of each fraction, 400 μl was counted as such after addition of 4 ml of Hionic Fluor (Wallac 1410 liquid scintillation counter, Pharmacia). Another aliquot of 400 μl was transferred to a weighed reaction vial, alkalinised with NaOH (pH–12) and kept in the oven at 70°C for 3 h to evaporate the acetonitrile and hydrolyse any esters present. After acidification (pH–3) with HCl, the volume was reconstituted to 750 μl and formate was determined as described before [8]. A [^{14}C]formate standard was taken along to determine the recovery. Recovery of [^{14}C]formate from [^{14}C]formyl-CoA was evaluated by using the synthesized [^{14}C]formyl-CoA standard and was comparable to the recovery of the formate standard (85%).

3. Results and discussion

In contrast to Sly et al. [11], we found that formyl-CoA is relatively stable at pH 3, but hydrolyses quickly at a pH above 5.5 even if kept at 4°C (Fig. 1). Therefore, incubations were terminated by acidification to pH 3 and reaction mixtures were kept on ice or at 4°C at all times after termination of the reaction, except during the actual HPLC analysis.

When purified peroxisomes were incubated with 3-methyl-[1-^{14}C]palmitate, HPLC analysis of the filtrate of the incubation medium revealed three ^{14}C-labelled peaks at approximately 2 min, 16 min and 27 min, respectively (Fig. 2). The first and the second peak coeluted with a formate and a formyl-CoA standard, respectively. Although difficult to see due to the low concentrations, the second and the third peak seemed to show a UV-absorbance at 254 nm and were alkali-sensitive. When the HPLC fractions were collected, hydrolysed and analysed for [^{14}C]formate, the counts of the first and second ^{14}C-labelled peak were recovered as formate (Fig. 3), confirming their identity as formate and formyl-CoA, respectively. However, no counts were recovered for the third peak (Fig. 3), suggesting the presence of an ester with a ^{14}C-labelled compound other than formate. The peak did not coelute with an acetyl-CoA standard (result not shown). The identity of this peak remains unknown at present. The amount of formate formed was 0.92±0.12 nmol/min per mg protein (mean±SE, $n=4$). Formyl-CoA and the unidentified compound both constituted 2–5% of the amount of formate.

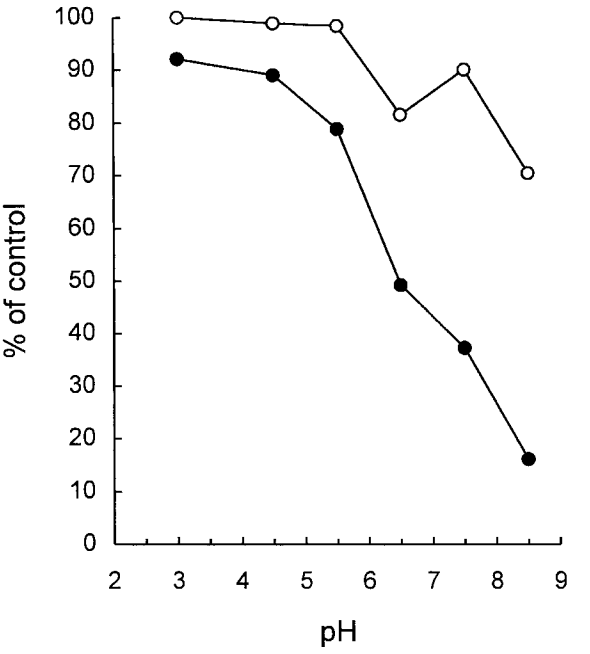

Fig. 1. Stability of formyl-CoA in function of pH. The degradation of formyl-CoA was measured over a pH range of 3–8.5. 20 nmol of synthetic [^{14}C]formyl-CoA were injected onto the HPLC after 5 (○) and 24 h (●) at 4°C in 50 mM of one of the following buffers: potassium phosphate (pH 3 and 6.5), potassium acetate (pH 4.5), 2-(*N*-morpholino)ethane-sulphonic acid (pH 5.5), Tris (pH 7.5) and Hepes (pH 8.5). Results are expressed as the % of formyl-CoA in a control sample injected at time 0.

To exclude the possibility that the formyl-CoA results from an activation of the formate produced during α-oxidation, purified peroxisomes were incubated with exogenously added [^{14}C]formate in the presence of the cofactors required for α-oxidation. No [^{14}C]formyl-CoA could be detected (results not shown), indicating that formate is not activated in peroxisomes. In contrast, when synthetic [^{14}C]formyl-CoA was incubated under these conditions with purified peroxisomes on the one hand and sucrose medium as a control on the other hand, 95.5% and 40.3% were recovered as [^{14}C]formate, respectively. Although before the incubation 20% of the label was present as formate (probably due to hydrolysis of formyl-CoA during storage), these results clearly demonstrate that

formyl-CoA is partly further hydrolysed due to the incubation conditions (37°C, pH 7.5), but that the major part of the formyl-CoA is actively converted to formate in peroxisomes, suggesting the presence of a formyl-CoA hydrolase (or CoA-transferase?) in rat liver peroxisomes. As far as we know, this is the first report on the presence in mammalian tissues of a formyl-CoA hydrolase, which up till now has been described only in microorganisms [11].

In conclusion, we found evidence that formyl-CoA is formed during α-oxidation of 3-methyl-substituted fatty acids in purified peroxisomes. The major part of formyl-CoA is actively converted to formate and the amount of formyl-CoA found constitutes only 2–5% of the amount of formate.

The formyl-CoA formed is not due to activation of formate — until now presumed to be the primary end-product of α-oxidation — but is rather than formate the end-product of α-oxidation. This makes it likely that the second end-product, the produced 2-methylbranched compound, is a fatty aldehyde rather than a fatty acid, as is generally believed [1].

An additional peak eluting later than formyl-CoA was seen, but contained no formate ester. Its nature remains unknown.

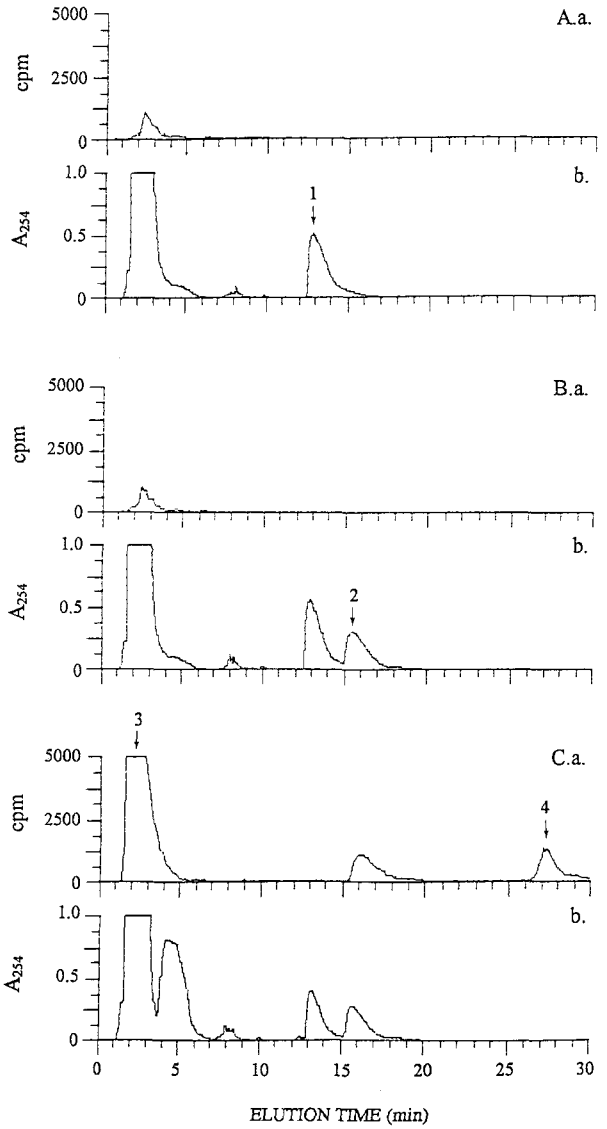

Fig. 2. HPLC analysis of the reaction medium after incubation of purified peroxisomes with 3-methyl[1-^{14}C]palmitate. Purified peroxisomes were incubated for 0 min (panels A and B) and 10 min (panel C) with 3-methyl[1-^{14}C]palmitate under α-oxidation conditions. After termination of the reaction by acidification, the reaction medium was ultracentrifuged and the supernatant filtered through a 10000 Da concentrator. An aliquot of the filtrate was analysed for [^{14}C]formyl-CoA by HPLC. HPLC effluents were monitored using an on-line UV-detector set at 254 nm and a radioactivity detector connected in series. Panels B,C: 45 nmol unlabelled formyl-CoA was added as a carrier. Each panel shows the radioactivity (trace a) and the UV-absorbance at 254 nm (A_{254}) (trace b). The radioactivity signal is delayed by about 0.2 min relative to the UV-signal. The numbered arrows indicate the elution of the following compounds: 1, CoA; 2, formyl-CoA; 3, formate and 4, unknown compound.

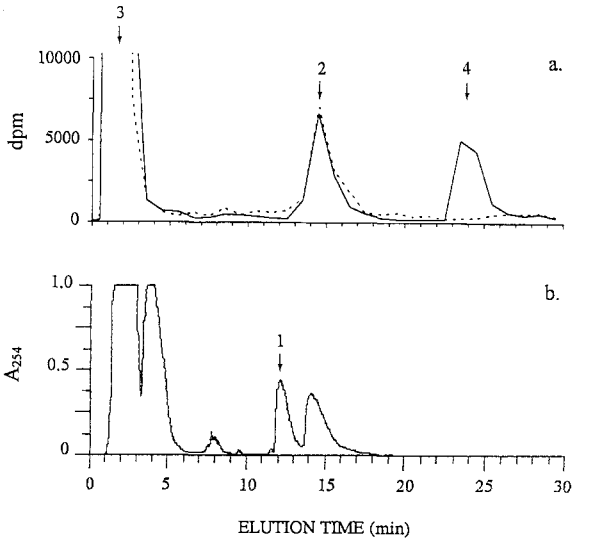

Fig. 3. Determination of formate on the HPLC effluents of the reaction medium after incubation of purified peroxisomes with 3-methyl[1-^{14}C]palmitate. Purified peroxisomes were incubated for 10 min with 3-methyl[1-^{14}C]palmitate under α-oxidation conditions and analysed by HPLC for formation of formyl-CoA as described in the legend to Fig. 2. HPLC effluents were collected after passing the UV-detector in fractions of 1 ml. Of each fraction, 400 μl was counted for radioactivity as such (panel a, full line) and another 400 μl was subjected to alkaline hydrolysis and after acidification used for the determination of formate (panel a, dashed line). Panel b shows the UV-signal at 254 nm (A_{254}). Arrows are numbered as for Fig. 2.

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